UNIVERSITY OF ALBERTA

Chemical characterization of growing antlers from wapiti (*Cervus elaphus*)

by

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The undersigned certify that they have read, and recommended to the faculty of graduate studies and research for acceptance, a thesis entitled **Chemical characterization of growing antlers from wapiti (*Cervus elaphus*)** submitted by **HOON-HEUI SUNWOO** in partial fulfillment of the requirements for the degree of

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DEDICATION

To GOD

To my parents

To my Son SeJun and Daughter SeIn, and my beloved wife MiSun

Who have consistently supported love and kindness

ABSTRACT

Antlers from deer species are alternative animal by-products. Due to the oriental trade, the velvet antler industry is rapidly emerging in North America. The unique property of antler with a deciduous natural phenomenon offers the valuable model of biomedical research. However, only limited information concerning biochemical and biological activities of growing antlers is available. Thus, studies were conducted to 1) investigate the chemical and morphological characterization of growing antlers of wapiti (*Cervus elaphus*), 2) elucidate the possible use of antler products as neutraceutical and/or pharmaceutical agents, and 3) characterize the biologically active components in growing antlers.

Growing antlers showed different chemical compositions according to cell populations consisting of mesenchymes, chondroblasts, chondrocytes and osseous tissues from distal to proximal portions of main beam. To explore the potential application of antler products in human health, their pharmacological effects on the immune response of growing rats were evaluated. Extracts of velvet antlers stimulated the bovine cell growth *in vitro* indicating the presence of growth promoting like substance for their pharmaceutical uses. Glycosaminoglycans, the possible active biological and pharmacological component were isolated, localized and characterized. Their structures were different from two tissues, cartilage and bone, in growing antlers. Proteoglycan as the intact form of glycosaminoglycans was also isolated in the zone of maturing chondrocytes where endochondral ossification occurs. Large aggregating proteoglycan and decorin turned out to be major proteoglycans.

In conclusion, new information obtained from this thesis will assist the deer industry in North America, stimulate the development of velvet antlers as nutraceuticals, functional food as well as pharmaceutical agents and warrant further physiological study on antler growth related to the biomedical research.

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Chapter 1.
General Introduction[1]

1.1. Introduction

Studies on antlers from deer family have fallen into two main sets: the physiological mechanisms of antler growth in the West, and the pharmacological activities of antlers in the Orient.

The extraordinary growth rate and unique deciduous nature of antlers has been explored since the time of Aristotle who recorded the effects of castration on antler growth more than 2,300 years ago. With such a long history of research, the study of antler growth and development began at the 19th century and is being considerably diversified to the biochemical, biological, pharmacological and physiological areas. Furthermore, the study on antler growth and development mechanism provides a unique model for biomedical research on pathological processes such as osteoporosis, atherosclerosis, cancer, wound healing and tissue implantation.

On the other hand, the use of antler for medicinal purposes was described in the representative ancient literature, Shen Nong Ben Cao Jing Herbal Classic Book tracing to the Han dynasty (206 BC - 220 AC) and DongEuiBoGam (1613) in Chosun dynasty. Since then, velvet antler has been used as a traditional medicine revealing convincingly medicinal efficacy for treatment of various diseases in oriental countries such as Korea, China, Japan and South Eastern Asia. From ancient to present, people in the zone of oriental culture have a belief of velvet antler to strengthen their body and mind.

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[1] *A version of this chapter has been submitted for publication. HH Sunwoo and JS Sim 1997. J. Nutra. Funct. Med. Foods.*

Velvet antlers as alternative agricultural by-products involve the most valuable material of traditional oriental medicine. Their medicinal purposes have been traded from China and Russia in the 19th century and from New Zealand and North America in 1970's to the Orient. Nowadays, the antler industry in North America is rapidly emerging due to increased demand and prices of antlers in international markets. Recently, production of velvet antlers around the world is expected to oversupply due to the steady of world consumption of antlers.

In order to increase the demand of surplus antlers in international markets, their use can be expanded in the West in obvious way, continuous increase of which is assisted by understanding of the mystic performance for the medicinal purpose. The continuing growth of the antler industry, therefore, heavily depends on the development and diversification of velvet antlers as both functional food and pharmaceutical agents through research and development. First of all, it is important to justify the use of velvet antler in traditional medicine by understanding its chemical and pharmacological properties. Therefore, all information generated from antler research is most crucial to substantiate velvet antler as nutraceuticals or medicinal food acceptable in the West.

1.2. What is Antler ?

Antler is defined as "the branched deciduous horn of any animal of the deer family" (Webster International Dictionary). The term 'antler' comes from the Latin 'anteoculae', meaning "in front of the eyes". In the orient, antlers refers to soft growing tissues, terms of "nog yong" in Korea", lu rong" in China, "rokujo" in Japan or "pantui" in Russia, and hard antlers before cast, terms of "lu jiao" in China or "nog gag" in Korea. Both soft and hard antlers are used for different purpose treating diseases in oriental medicine.

Antlers are appendages of the skull, composed of a solid bony core and supported on skin covered pedicles (protuberance of the frontal bone) which are permanent tissues. The formation of primary antlers from males except reindeer can be initiated after pedicle development from the periosteum of the frontal bones of almost all members of *Cervidae* (Goss 1983). While the antler is growing or regenerating, it is covered by a skin with a dense of fine hair that leads to the term "velvet antler" which can be applied to the growing tissue. After growth is finished, the velvet skin peels off to be known as hard antler.

Antlers differ from the keratinous horns of the Bovidae in that horns are not cast and regrown annually, while antlers are deciduous with a cycle of regeneration, proliferation, mineralization and casting off. Growing antler has fine hair known as velvet. Cervids such as caribou, wapiti and moose inhabiting North America have antlers, while bovids including mountain goat, bighorn sheep, bison and pronghorn antelope possess horns. With the exception of caribou (*Rangifer tarandus*), antlers are found only on males. A small proportion (about 1%) of female deer grow antlers because of excessive testosterone. On the other hands, horns are permanent structures composed of a bony core and an outer sheath of keratin, the same material found in fingernails and hooves. The horns of bovids never branch, whereas antilocaprid (pronghorn) horns have a forward-projecting prong. In North American bovids, horns are found on both genders, though they are smaller on females.

The *Cervidae* (family in taxonomy) is currently classified into 4 subfamilies: *Cervinae* with four genera (Axis, Cervus, Dama, and Elaphurus), *Hydropotinae* with one genus (Hydropotes), *Muntiacinae* with two genera (Elaphodus and Muntiacus) and *Odocoileinae* with nine genera (Alces, Blastocerus, Capreolus, Hippocamelus, Mazama, Odocoileus, Ozotoceros, Pudu and Rangifer). In Oriental classification, animals for the traditional use of antlers are limited only to *Cervus* species (*Cervus nippon and Cervus elaphus*) according to the Oriental Countries' Specification for natural medicines.

The family *Cervidae* is represented in North America by the elk (derived from the Germanic "elch", which refers to the European moose) or wapiti (a Shawnee Indian name meaning "white rump") (*Cervus elaphus*), moose (an Algonkan Indian word for "eater of twigs") (*Alces alces*), caribou (a Micmac Indian name, which refers to reindeer) (*Rangifer tarandus*), black-tailed deer (*Odocoileus hemionus*) and white-tailed deer (*Odocoileus virginianus*). This family dates from the Miocene in the Old World except the genus *Odocoileus* in the New World (Geist 1981). Wapiti, moose and caribou in North America have counterparts in Europe and Asia. There are no European or Asian counterparts to the genus *Odocoileus*. The wapiti is a common name of the American elk larger than the European red deer, which exceeds several-fold in size (Goss 1983). The calves grow their first antlers with unbranched spikes as yearlings. The bulls (adult wapiti) cast their hard antlers in March or April during the Northern light photoperiod, and reproduce them immediately. The velvet (highly innervated skin covering the growing antler) is shed in August and then hard antlers remain until casting.

A number of theories of antler function have been proposed. During the rutting season, they are used in threatening or intimidating behavioral function to rivals (Lincoln 1972) and in sparring contests (Bubenik 1968). On the other hand, antlers function as display organs, determining social hierarchy among males without actual battle. As status symbols, they dictate the rank order in bachelor groups which determines priority for food and shelter (Bubenik 1968). It has been suggested that antlers evolved as thermoregulatory organs (Stonehouse 1968). Although antlers may have some role in heat dissipation (the growing tips of antlers are often the warmest parts of the body), a primary evolutionary role is refuted by the fact that Roe deer (*Capreolus capreolus*) and some tropical deer grow their antlers in winter (Geist 1968). The olfactory projection theory (Bubenik 1968) postulates that antlers dissipate pheromones from rich sebaceous glands in velvet to mark boundaries in territorial species.

1.3. Growth of Antler

The formation of primary antlers is initiated after pedicle development from the periosteum of the frontal bone. Antler growth occurs at the tip containing reserve mesenchyme which can be proliferated and differentiated into chondroblasts and chondrocytes responsible for the formation of cartilage, followed by maturation, hypertrophy and calcification. The growth of antler for approximately 4 months is suspended with the increase of massive mineralization of the bone, followed by shedding the velvet skin. The hard antlers are retained during rutting season and winter period until the time of casting. Mature antlers are composed of dead bone, comprising a thick outer layer of compact bone surrounding a central core of cancellous bone. Antler growth is seasonal in boreal and temperate species of deer. For example, in the seasonal wapiti (*Cervus elaphus*) the velvet antler grows in spring and is hard for breeding season in autumn. Previous antlers are cast in spring. The seasonal cycle of regeneration, differentiation, mineralization and casting of the previous set of hard antlers is closely related to the change of day length and reproductive cycle of the male deer regarding hormones such as melatonin, testosterone, estrogen, thyroxine, prolactin, growth hormone, cortisol and insulin growth factor (Bubenik et al. 1974, 1975; Sempere and Boissin 1981; Morris and Bubenik 1983; Goss 1983; Brown et al. 1983a, b; Dinsmore et al. 1986; Reyes et al. 1993; Lewis and Barrell 1994; Lincoln and Tyler 1994; Suttie et al. 1995).

The growth of antler depends on the endochondral ossification in length and the intramembranous ossification in diameter (Banks 1974). In the former, cartilage first calcifies to form bones in perpendicular growth, while mesenchymes in periosteum become osteoblast and then bones in horizontal growth. To understand the development of the rapidly growing antler which is approximately 2.75 cm/day in the case of elk (*Cervus elaphus*) (Goss 1970), there are histological observations related to the growth of antlers of various breeds (Modell and Noback 1931; Wislocki 1942; Wislocki et al. 1947; Mollelo et al. 1963; Banks 1974; Banks and Newbrey 1983; Rönning et al. 1990; Li and Suttie 1994; Kierdorf et al. 1994; Szuwart et al. 1994, 1995; Price et al. 1996) on the ossification compared to the long bones.

Antler growth is associated with minimal seasonal levels of testosterone. Rapid mineralization is then associated with a slow rise of testosterone levels. Increases of androgens in blood (occurring several months before the rut) result in shedding of velvet and the death of antlers. In the end of the cycle, a reduction of testosterone levels after the rut is manifested by the casting of antlers. Epidermal growth factor has been found in relatively high concentrations in velvet of antler (Ko et al. 1986). Extensive studies on insulin-like growth factor (IGF) have been related to antler growth (Elliott et al. 1992, 1996;. Sadighi et al. 1994; Price et al. 1994; Suttie et al. 1989, 1991, 1995). It has been known that IGF is not locally produced in antler tissue but acts in an endocrine way on antler growth. It has been suggested that IGF is stimulatory for antler and body growth in red deer (Suttie et al. 1988).

Other growing tissues contain a great amount of growth factors such as epidermal growth factor, fibroblast growth factor, nerve growth factor, platelet-derived growth factor, tumor necrosis factor, transforming growth factor, α and β, and others which promote cell-cell interaction (Centrella and Canalis 1985). Thus, several other growth factors little studied in the velvet antler have effects on antler growth and development. The study of thier possible role has to be conducted to understand the antler growth.

1.4. Pharmacological Efficacy of Antler

Velvet antlers have been traditionally used with a strong belief to enhance the physiological function of our body as a tonic and treat diseases in oriental medicine. In the Orient, antler has been used as a traditional herbal medicine with a mixture of other herbs. Oriental medicine emphasizes the synergistic effects of herbs for history exceeding 2,000 years. The use of antler for medicinal purposes dates to back to the Han dynasty (206 BC - 220 AD) in which Shen Nong Ben Cao Jing Herbal Classic book was described. A historical Korean literature, DongEuiBoGam (1613), defined antlers as soft growing tissues with velvet for the medicinal use. Ancient books described the efficacy of antler for treating impotence, menoxenia, dysfunctional uterine bleeding, dizziness and vertigo, insomnia, amnesia, and arthritis. Interestingly, Western literature also showed the use of antlers as follows: the Roman Plinius Secundus wrote that antlers contained "some sort of healing drug" and described its use for treatment of epilepsy in the first Century AD. Topsell (1607) described the use of powdered antler to treat baldness, pimples, lice, toothaches and snakebite.

Velvet antlers have been traditionally used as boiling-water soluble extracts (WSE) and alcohol extracts (AE). Animal studies have evaluated the efficacy of WSE in: improving hepatic oxidative phosphorylation and decreasing cholesterol content on liver tissue of rabbits (Yong 1964); activation of hepatic enzymes in starved rats (Lee 1980); effects on liver detoxification (Choi et al. 1979); increase of erythropoietic activity in rabbits (Song 1970) and rats (Kim and Park 1982). Ko and Song (1986) reported that oral administration of WSE increased the number of T- and B-lymphocytes and the natural killer cell activity in mice. Li and Wang (1990) reported that the treatment of rats with AE results in marked increases in the number of monocytes which are related to the immune system. Wang et al. (1985) reported that polysaccharides extracted from velvet antler have an anti-ulcer effect. Hot water extracts obtained from antler of *Cervus nippon* showed anti-aging effects in male senile animals with the increase of testosterone levels (Wang et al. 1988a). The WSE also enhances hemoglobin production and increases hematocrit (Bae 1976). Zhao et al. (1992) reported the presence of anti-complementary activity in the antler proteoglycan which is made up of mainly chondroitin sulfate isolated from antler. The WSE showed an inhibitory effect on monoamine oxidase-B activities in the liver and brain of aged mice (Chen et al. 1992). Preliminary data from our laboratory also showed that the intraperitoneal administration of hot water extract obtained from wapiti antler to mice resulted in an increased growth rate. Further investigation of the active substances with growth promoting activity in growing antler extracts is in progress in our laboratory. Wang et al. (1990) reported that antlers contain polyamines such as putrescine, spermidine and spermine. Their effects stimulated protein and RNA synthesis in tissue culture. Bubenik (1990) mentioned that an extract from the growing antler tip section facilitates healing of epidermal wound on the dorsum of albino rats. Water soluble glycosaminoglycan rich extract from cartilaginous tissues of wapiti antlers was examined on the growth of bovine skin fibroblasts in culture (Sim et al. 1995a,b; Sunwoo and Sim 1996) and showed growth-promoter like activity (Sunwoo et al. 1997). This kind of experimental information is useful to develop the pharmaceutical agent from velvet antlers.

Alcohol extract of antlers has been investigated by researchers from Russia, Japan, and China. Pantocrin treatment enhanced glycolysis in nervous tissue, an actual effect specific to neural tissue (Takikawa et al. 1972a,b). A number of reports from Russia are concerned with Pantocrin (alcohol extract of antler from or Rantarin which is ethanol extract. Hypotensive effects of alcohol extract from antler is due to the presence of lysophosphatidylcholines with C10:0, C20:0, and C16ω:1 fatty acids (Tsujibo et al. 1987). Pantocrin has a hypotensive effect in animals under anesthesia although it is transient (Fennessy 1991). Wang et al. (1988b) studied the effect of ethanol extract on the recovery of rats from the hepatic lipid peroxidative injury induced by free radical oxygen, and reported that the extract has anti-lipid peroxidation activity. Bae (1975) found that feeding antler powder to broiler chickens results in an increase in growth rate and feed efficiency and AE decreases the blood pressure of chickens (Yudin and Dobryakov 1974). We have also confirmed high hematocrit in laying hens fed dried wapiti antler powder (H. H. Sunwoo and J. S. Sim unpublished results).

In extensive *in vivo* and *in vitro* studies, antler preparations convincingly show that velvet antler contains an active component(s) which influence(s) body metabolism in general (stimulating and tonic), protect and restore damaged organ tissues (accelerating healing/recovery from injury), promote immune and phagocytic functions (anti-inflammation, anti-arthritis, anti-stress), slow the aging process, have hypotensive-vascular effect, and enhance gonadotrophic and thyroid functions. However, the representative and principal components in antler extracts are still unclear.

1.5. Chemical Characterization of Antler

Chemical characterization of velvet antlers has been performed to understand the possible active components to elucidate their medicinal efficacy (Table 1.1.). The main components of antlers were known to contain amino acids such as glycine, alanine, proline, and glutamic acid in majority, histidine, isoleucine, leucine, serine, tyrosine and lysine in minority, fatty acids, phospholipids and elements of Ca, P, Mg, Fe, K, and Na as major minerals, Ni, Cu, Ti, Mn, Sn, Pb, Si, and Ba in small amounts.

Alcohol extracts of velvet antlers were analyzed. Elyakov and Cherezova (1968) extracted ceramide in sika deer antlers. Lysophosphatidylcholine with palmitic acid (C16:0) accounted for 50% of total fatty acids was isolated from growing antlers (Tsujibo et al. 1987). Sim and Hudson (1991) suggested that high and low sections of wapiti growing antler have different lipid contents and fatty acid profiles. Han and Jhon (1992) reported the presence of sialic acid-containing glycolipid in antlers. Ivankina et al. (1993) found that lipid compositions containing prostaglandin, phospholipids and polyunsaturated fatty acids changed at different stages of antler growth. Lecithin was detected in antler extracts (Yamasaki et al. 1994).

Frasier et al. (1975) reported the presence of hyaluronic acid and chondroitin sulfate in growing antlers through microscopic observations. Velvet layer also contained mucopolysaccharides of hyaluronic acid and chondroitin sulfate isolated by papain digestion (Kim et al. 1976). Scott and Hughes (1981) found the presence of chondroitin sulfates in fossilized antler. Zhao et al. (1992) reported the complement activity of glycoprotein which is mainly composed of glycosaminoglycans in antler *(Cervus nippon)*. Antlers contain bone morphogenetic protein 2 and 4 (Feng et al. 1995; 1997).

Velvet antler would be a rich source of growth-promoter like activities due to the nature of antler growth. Epidermal growth factor has been isolated from the velvet layer of growing antler (Ko et al. 1986). Tip sections (distal portion of growing antler) may contain growth factor-like molecules which are glycoprotein with chondroitin sulfate and keratan sulfate to stimulate the growth of bovine skin fibroblasts (Sunwoo and Sim 1996).

There are reports on the study of molecules related to the extracellular matrix components which are composed of primarily collagen, elastin, structural glycoproteins and proteoglycans in velvet antlers (Figure 1.1.). Currently, four types (I, II, III and X) of collagens have been identified and immunohistologically localized in antlers (Speer 1983; Newbrey et al. 1983; Price et al. 1996; Gibson et al. 1996) as a marker of the chondrocyte phenotypes including large aggregating proteoglycans which is the most abundant molecule in extracellular matrix of cartilage (Doege et al. 1991). However, proteoglycans as the second family of extracellular matrix components are not well understood in growing antlers. In general, large group of structural glycoproteins including fibronectin, laminin, undulin, nidogen, tenascin, vitronectin and osteonectin are present in the extracellular matrix, basement membranes, at the cell surfaces and even in subcellular compartments, but unknown in antler.

Above information involves the broad chemistry of velvet antlers to find out their active components as biological and physiological roles. Whatever data show, a solid set of chemical data obtained from velvet antlers is important to explain the medicinal efficacy, to develop the pharmaceutical agents and to use them as food supplements for human consumption.

1.6. Antler Industry

Velvet antlers have been an important component of traditional Oriental medicines for many centuries. With increasing demand of velvet antler, deer farming has been becoming a popular alternative form of animal agriculture in many parts of the world including North America, Europe and New Zealand (Sim 1987). Thanks to Oriental consumption, World production has increased about 30% per year stimulating rapid growth of deer farming around the world. World wide, about five million deer are farmed, most of which serve the dual purposes of venison and antler production.

North American elk and deer farming is a rapidly maturing industry with considerable potential in the international marketplace. With a short history, deer farming in Canada commenced as a viable industry in 1970. Canada, with over 70,000 elk on farms, holds almost 60% of the North American population and has become an important world source for breeding stock and antler products (slice, powder and extract). In 1995, Canadian deer farms produced an estimated 15 metric tonnes of velvet antlers for export to Korea which accounts for approximately 85% of the amount of velvet antlers circulated in international market. Major countries as antler-exporter to Korea are China, New Zealand, Russia and North America (Figure 1.2.). It is estimated that the Korean market will import about 200 metric tonnes of dry velvet antlers per year (Figure 1.3.). The amount of import may decrease due to the economic deadlock.

Seemingly, world consumption has remained static, or has decreased. Surplus antler stock is expected in the traditional world market within 5 years. However, the opportunity for development of a modern nutraceutical market appears to be vast with strong demand in North America and Europe as well as in Asia. Despite the current economic opportunity, prospects for developing a western nutraceutical market have been dampened by incomplete understanding of the chemical and pharmacological properties of velvet antler. The key to survive the antler industry depends on the research and development of antler

1.7. Antler Processing

The antler industry is seeking a marketing strategy to create the demand to cope with oversupplies of antlers in traditional markets. Alternative way is to diversify antler products as food supplements or functional food through proper processing technology based on the chemical and pharmacological knowledge. In the past, harvested antler shipped to international market under a frozen condition for only Oriental trade. Antlers were processed by its own method and then recirculated with a high margin. Thus, the processing method has been developed by antler suppliers.

Traditionally, the purpose of processing is to keep antler from deterioration for a long time storage and to conserve the shape and size for the demand of market. In general, hot air drying method is still popular among producers of antler. The harvested antler hangs in a drying room with circulation of air (approximately 60°C), turned down and up several times a day for many days until well dried. The velvet covering is then removed by scraping and burning. The dried antler is cut into sections, placed in alcohol for about a day following evaporation and then sliced thinly. Chinese processing method removes blood in velvet antler by a vacuum, dips the antler into hot and cold water repeatedly for several times to drain residual blood, and then dries it by air. Pantocrin and Rantarin by Russians are derived from alcohol extracts of antlers from sika deer, maral deer and wapiti, and reindeer, respectively. New Zealand has developed a cooking method by steam of frozen velvet antler above 100°C followed by electric dehydration for about 30 days to keep about 15% of its water contents (Won 1994).

Traditional markets require high quality of antler determined by shape, size, and mineralized conditions. The harvested antler contains cartilaginous, mineralizing and mineralized tissues. The quality of antlers evaluated in international markets might be different depending on breeding areas, nutrition and management, harvesting time and processing methods. For example, mineralization seems delayed at extreme latitudes. Weight of antler increases modestly in good nutritional and managemental conditions (Moore et al. 1988). Optimal harvesting time is also important to satisfy requirement of market in that the circulated antler keep tissues not to be mineralized according to old literature and perception of consumers. In New Zealand, antlers from red deer are harvested at which the royal tines began to appear 55-70 days after the casting of the previous antler (Fennessy 1991). Wapiti antlers are harvested in June in northern photoperiods at 60-70 days of growth (Sunwoo et al. 1995).

1.8. Ethics and Industry

The future of the velvet antler industry turns as much on issues of animal welfare as product quality. The traditional velvet market demands antler cut at 60-70 days from button-drop. At this stage, growing antler is highly innervated and vascularized (Woodbury and Haigh 1996). The Canadian Code of Humane Practice for Farmed Deer (1997) expresses concern about procedures for velvet removal at this early stage and calls for research on alternatives to chemical anesthesia and attendant residue problems. However, there is an alternative approach. If active components can be identified, extracted and concentrated, antler removal perhaps could be delayed until more than 100 days when antler hardens, obviating significant welfare and drug residue issues. Even in Britain where velvet antler removal is banned, hard antlers are removed before the rut to prevent injury among rutting stags and damage to fences and facilities (Haigh and Hudson 1993).

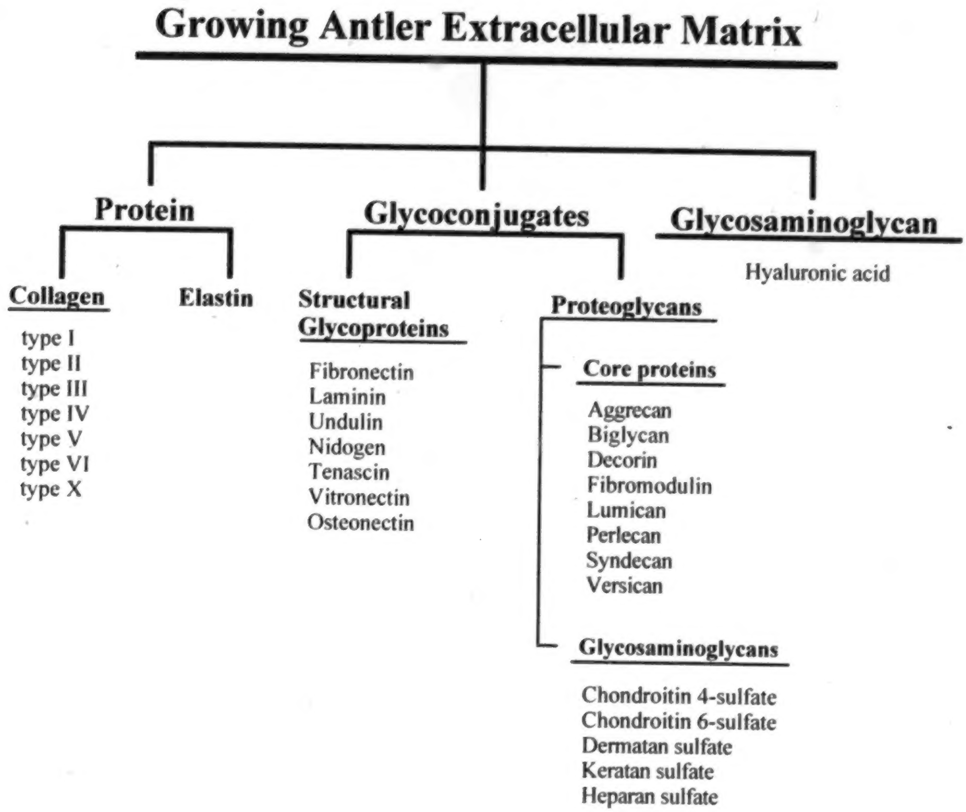
1.9. Objectives of Thesis

Since deer farming boomed in the past decades, velvet antler has been developed for the production of value-added products believed to contain biologically active components. The belief on velvet antlers has been continued for many centuries in the Orient as the most valuable folk medicine which shows mystic performance in the preventative and/or curative effect on human health. However, few information is available concerning the biological activities in the West. Overall the research will accumulate the knowledge to understand the antler chemistry and bring benefits to antler industry in Canada.

First of all, as shown in Figure 1.4., the approach of antler research in this thesis was started to understand histology and chemistry of wapiti antlers in growing stages demanded by the international market. The present thesis also includes studies on the pharmacological efficacy of antlers based on their Oriental uses for human consumption. The ultimate aim of this research is to characterize biologically active component(s) related to rapid growth of antler for the purpose of developing neutraceutical and/or pharmaceutical agents. Thus, this information will boost the potential of the velvet antler industry in Canada and promote the biological research on antler growth.

Table 1.1. General components of velvet antlers

Minerals	Amino Acids	Lipid	
		Fatty Acids	Phospholipids
Calcium	Aspartic acid	C9:0	Phosphatidylethanolamine
Phosphorus	Threonine	C10:0	Phosphatidylcholine
Magnesium	Serine	C11:0	Phosphatidylinositol
Sulfur	Glutamic acid	C12:0	Phosphatidylglycerol
Sodium	Proline	C12:ω1	Phosphatidyldiglycerol
Potassium	Glycine	C14:0	Phosphatidylserine
	Alanine	C15:0	Lysophosphatidylethanolamine
Trace Minerals	Valine	C15:ω1	Lysophosphatidylcholine
Manganese	Methionine	C16:0	Sphingomyeline
Zinc	Isoleucine	C16:ω1	Phosphatidic acid
Copper	Leucine	C17:0	
Selenium	Tyrosine	C17:ω1	
Cobalt	Phenylalanine	C18:0	
Iron	Lysine	C18:ω1	
	Histidine	C18:2ω6	
	Arginine	C18:3ω3	
	Tryptophan	C20:0	
	Cysteine	C20:2ω6	
		C20:4ω6	



Growing Antler Extracellular Matrix
Protein
Glycoconjugates
Glycosaminoglycan
Hyaluronic acid
Collagen
type I
type II
type III
type IV
type V
type VI
type X
Elastin
Structural Glycoproteins
Fibronectin
Laminin
Undulin
Nidogen
Tenascin
Vitronectin
Osteonectin
Proteoglycans
Core proteins
Aggrecan
Biglycan
Decorin
Fibromodulin
Lumican
Perlecan
Syndecan
Versican
Glycosaminoglycans
Chondroitin 4-sulfate
Chondroitin 6-sulfate
Dermatan sulfate
Keratan sulfate
Heparan sulfate

Figure 1.1. Extracellular matrix components of growing antlers.

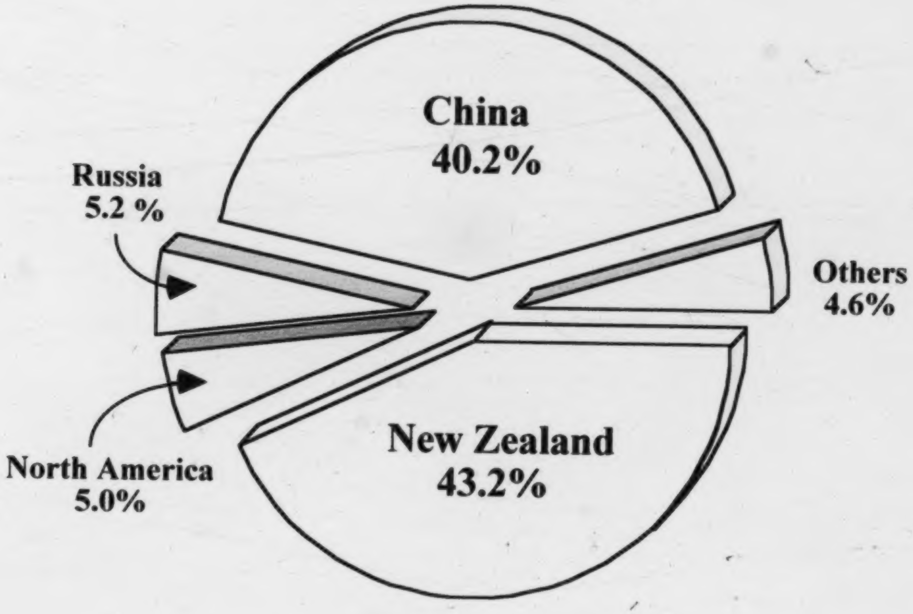


Figure 1.2. Major countries exporting velvet antlers to Korea in 1996.

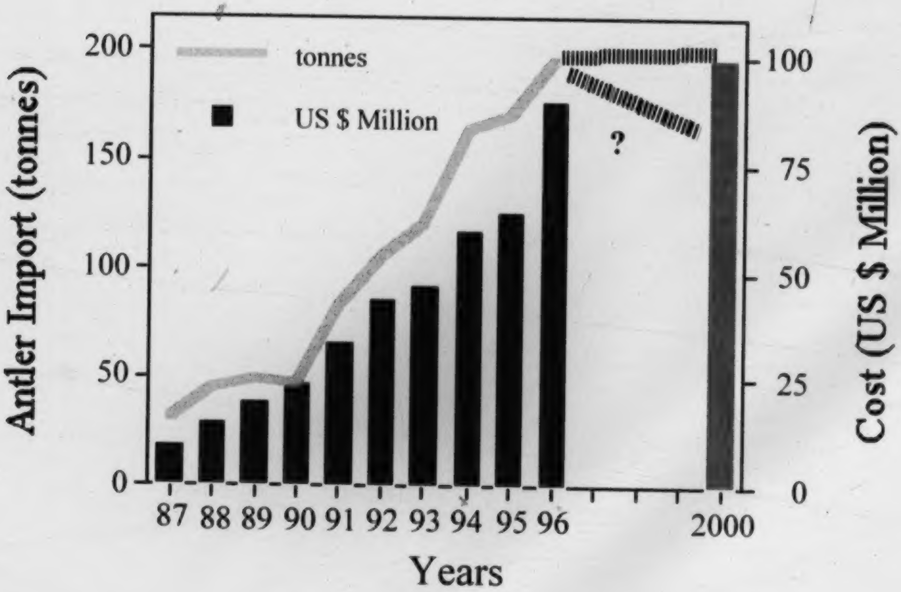


Figure 1.3. Importing amount of velvet antlers in Korea (1987 - 1996).

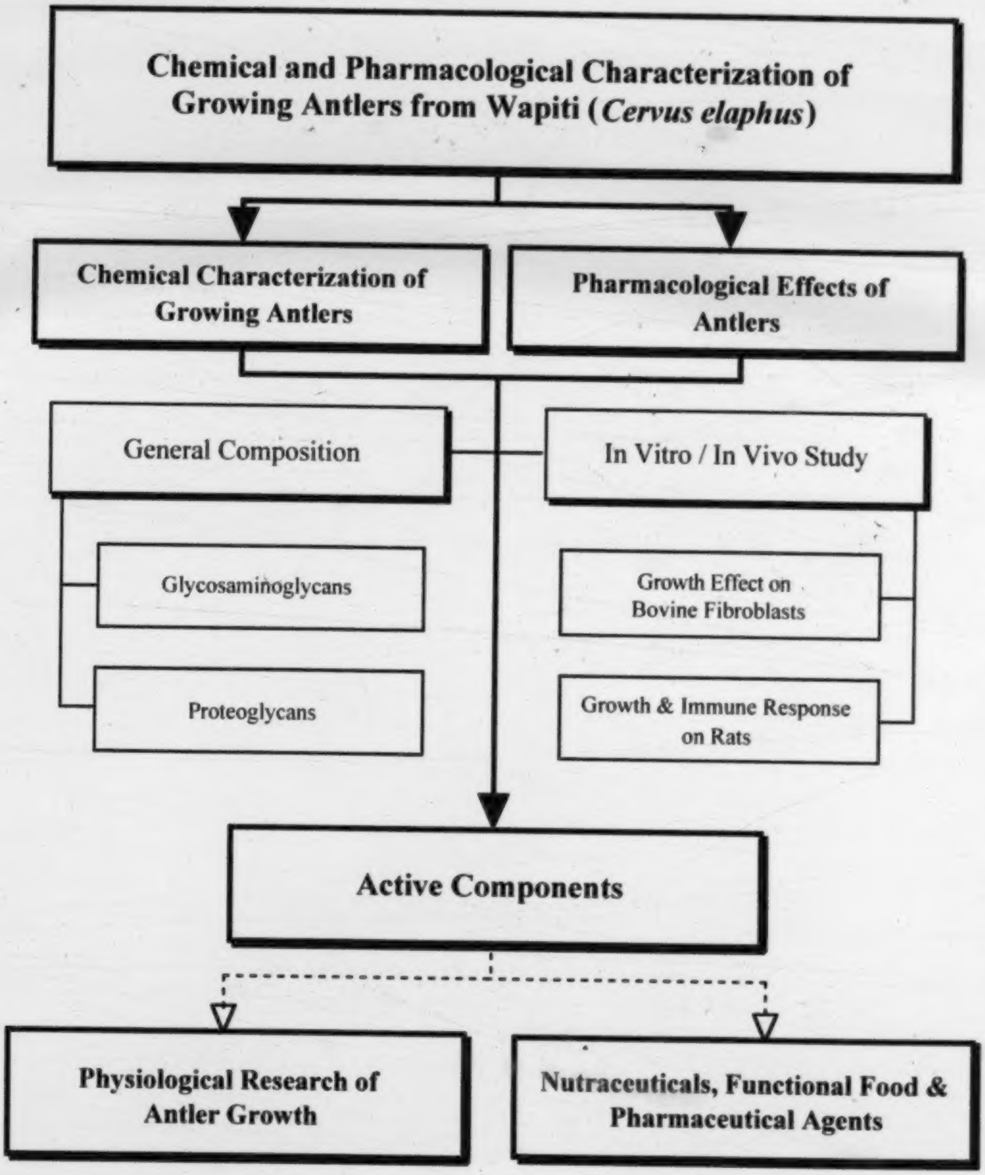

Chemical and Pharmacological Characterization of Growing Antlers from Wapiti (*Cervus elaphus*)
Chemical Characterization of Growing Antlers
Pharmacological Effects of Antlers
General Composition
In Vitro / In Vivo Study
Glycosaminoglycans
Proteoglycans
Growth Effect on Bovine Fibroblasts
Growth & Immune Response on Rats
Active Components
Physiological Research of Antler Growth
Nutraceuticals, Functional Food & Pharmaceutical Agents

Figure 1.4. Flow diagram of antler research.

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Chapter 2.

Histological Study of Growing Antlers of Wapiti (*Cervus elaphus*)

2.1. Introduction

The modern use of antlers as a functional food is expanded into North America and Europe as well as in Asia. International markets demand velvet antler of which quality is determined by specific size, color, shape and mineralization. Thus, antlers of wapiti (*Cervus elaphus*), one of Alberta's cervid species, are harvested at 60-65 days of regrowth (June in Northern photoperiods) following the casting of the previous set of hard antlers. The harvested antler in a growing stage is composed of soft cartilaginous tissues in distal portion and hard bony structures downward.

Antlers grow in length by endochondral ossification, and in diameter by intramembranous ossification (Banks 1974). Antlers are probably the fastest growing non-pathological tissues among mammals. For example, the maximum growth rate of antlers of elk (*Cervus elaphus*) is approximately 2.75 cm/day (Goss 1970). They are suggested to be a useful model for the study of bone growth and metabolism (Goss 1983). The growth of antler depends on the proliferation and maturation of chondrocytes that occur in the tip and upper, respectively (Banks and Newbrey 1983). Antlers can be divided into four sections, tip, upper, middle and base which are used differently to treat diseases such as anemia, arthritis, hypercholesteremia, and cancer or to promote health as traditional oriental tonics (Fennessy 1991).

However, little is known about the histology of wapiti (*Cervus elaphus*) antler circulated in markets. The present study was carried out to investigate cell morphology in main beams of the harvested antlers of wapiti (*Cervus elaphus*) by using light microscopy.

2.2. Materials and Methods

Samples of fresh antlers were obtained in June at 65 days after button-casting from four 4 year old wapiti (*Cervus elaphus*) kept at the University of Alberta Ministik Research Station. Care of animals and procedures used were in accordance with the guidelines of the Canadian Council on Animal Care (1993). Cylindrical, longitudinally-oriented biopsy samples were taken from distal to proximal portions of the main beam. They were fixed in 4% buffered formalin, pH 7.3 containing 0.5% cetylpyridinium chloride (Williams and Jackson 1956).

All fixed samples were decalcified in a solution containing 22.5% formic acid and 10% sodium citrate. After dehydration by passage through an ascending alcohol series, the specimens were embedded in paraffin and sectioned at 5 μm. The sections were deparaffinized in xylene, hydrated in a alcohol series of increasing concentrations, and stained with Hematoxylin and Eosin (Drury and Wallington 1967).

2.3. Results and Discussion

Histological observations have been reported on the cell morphology of rapidly growing antler (Modell and Noback 1931; Wislocki 1942; Wislocki et al. 1947; Mollelo et al. 1963; Banks 1974; Banks and Newbrey 1983; Li and Suttie 1994; Kierdorf et al. 1994; Szuwart et al. 1994, 1995; Price et al. 1996). In the present study, the main beam of a growing antler of wapiti (*Cervus elaphus*) can be divided into four major zones by cell populations through microscopic observation (Figure 2.1.). The distal tip section (approximately 1 - 1.5 cm from top of the antler) contained the distinct region (Figures 2.2.a - 2.2.d) including those of reserve mesenchymes adjacent to the dermis, prechondroblasts, chondroblasts and immature chondrocytes. The cellularity was apparently highest in the zone of prechondroblasts, and gradually decreased as cells matured. In the zone of prechondroblasts, both cell proliferation and development of eosinophilic structures appeared to be active (Figure. 2.2.b). These structures had relatively low cellularity, and were oriented parallel to the long axis of the main beam of antler. They appeared to be precursors of perivascular space. The perivascular area, which was relatively small in the zone of chondroblasts, increased downward. The zone of tip section in antlers is a growth center by development with the proliferation and differentiation of mesenchymal cells into chondroblasts and chondrocytes, followed by cartilage maturation, hypertrophy and calcification in upper section, downward.

The upper section containing chondrocytes as major tissues and primary spongiosa as minor tissues was comprised of columns of clustered maturing chondrocytes separated by perivascular spaces containing blood vessels. The size of chondrocytes, which was larger than that of chondrocytes in the tip sections, increased as cells mature. Matured hypertrophied chondrocytes adjacent to perivascular spaces were found to lose nuclei staining with Hematoxylin (Figure 2.3.). These cells appeared to be calcifying chondrocytes. Osteoblasts were seen adjacent to the calcifying cartilage (Figure 2.3.). The number of calcifying chondrocytes increased in proximal direction with concomitant decrease of the area of cartilaginous tissue. These observations suggested the occurrence of endochondral ossification undergoing development of cartilage, calcification, and osseous replacement, sequentially. From this study, cells in the tip section still proliferate into cartilaginous tissues and mature chondrocytes in upper sections were observed to calcify. The present findings are also in agreement with the idea that the antlerogenesis requires endochondral ossification and intramembranous ossification for the longitudinal growth and the circumferential growth, respectively (Banks 1974). The process of endochondral ossification includes several events including growth, maturation and calcification of chondrocytes followed by invasion of bone forming cells, osteoblasts (Figure 2.3.).

The boundary between upper and middle sections shown in Figure 2.1. is not clear. In the present study, the middle and base sections contained bony structures including spongy bone with osteoid on its margin (Figure 2.4.a), and cortical bone structure (Figure 2.4.b). The cortical bony tissues were covered with tissues of cellular periosteum containing mesenchymal cells, which differentiate into bone forming cells, osteoblasts (Jee 1983). The difference of both sections is a degree of mineralization and is a thickness of cortical bony tissue adjacent to outer velvet layer. The middle section includes the stage of mostly primary spongiosa in calcifying zones, while the base section dissected at approximately 3 cm from pedicle was observed to contain thicker cortical bony tissues than the middle section did, indicating hardening of tissues by calcium accumulation. The intramembranous ossification is dependent on the differentiation of mesenchymal cells into osteoblasts in the cellular periosteum and is responsible for latitudinal growth of periphery of main beam in growing antlers (Figure 2.4.). Osteoblasts form osteoid tissues which are subsequently mineralized to form bone spicules. In general, the growth of antler is suddenly suspended with the increase of massive mineralization after approximately 4 months of regrowth. The hard antlers are retained during rutting season and winter period until the time of casting.

The light microscopic observations of antlers of wapiti in this study were consistent with the report of Banks (1974) who studied white-tailed deer (*Odocoileus virginianus*) antler and reported that antlerogenesis requires both endochondral ossification and intramembranous ossification. We did not find any clear evidence to support Rönning et al. (1990) who reported direct conversion of cartilage into bone (by the merge of chondrocytes into bone) without any signs of endochondral ossification. The process of endochondral ossification involves several events including growth, maturation and calcification of chondrocytes followed by invasion of bone forming cells, osteoblasts. The intramembranous ossification is dependent on the differentiation of mesenchymal cells into osteoblasts in the cellular periosteum.

In the Orient, different portions have been used for particular purpose to treat diseases in the modern preparation of antler (Fennessy 1991). For example, it might be of importance to understand why the traditional use of actively growing soft portions of antler has been focused for children and young people as a preventative medicine. From histological observations of growing antler, the tip and upper sections contain more blood vessels and cartilaginous tissues. Also, the mineralizing middle sections have been used in the treatment of arthritis and osteomyelitis, while the mineralized base section in old people lacking calcium. However, the chemical composition of each section has not been elucidated.

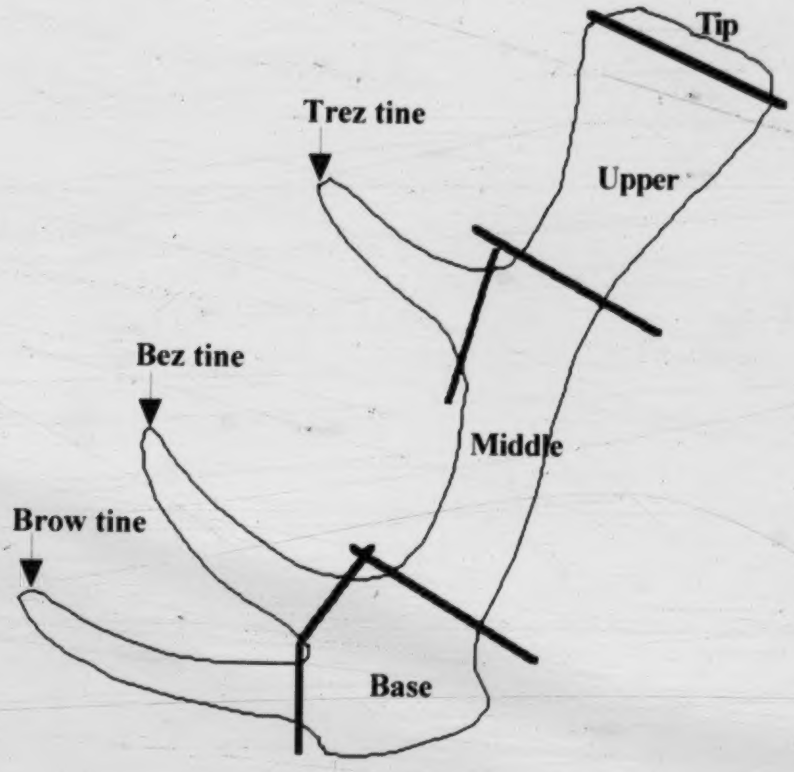
Tip
Trez tine
Upper
Bez tine
Middle
Brow tine
Base

Figure 2.1. Optimal stage of velvet antlers of wapiti demanded by international markets showing four sections used in current research.

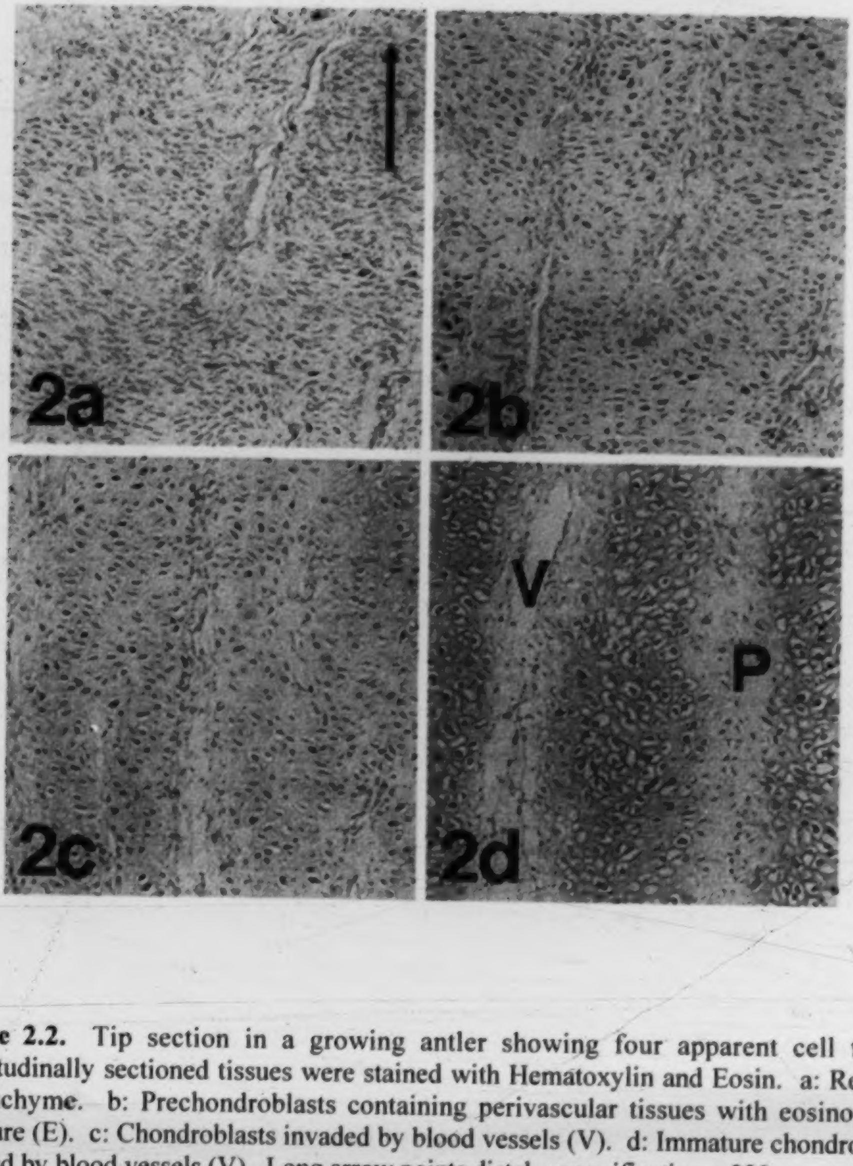

Figure 2.2. Tip section in a growing antler showing four apparent cell types. Longitudinally sectioned tissues were stained with Hematoxylin and Eosin. a: Reserve mesenchyme. b: Prechondroblasts containing perivascular tissues with eosinophilic structure (E). c: Chondroblasts invaded by blood vessels (V). d: Immature chondrocytes invaded by blood vessels (V). Long arrow points distal. magnification x 330.

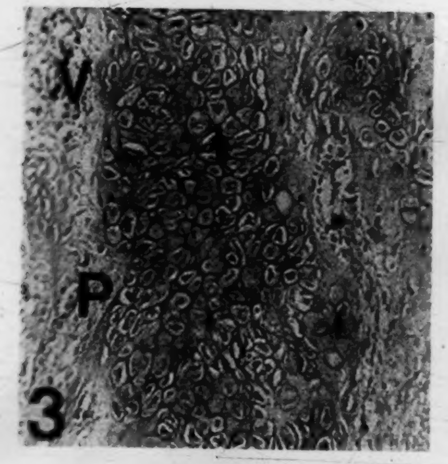

Figure 2.3. Upper section in a growing antler showing matured hypertrophic chondrocytes (large arrow). Calcifying chondrocytes (small arrow) and lining osteoblasts (arrow head) adjacent to the perivascular space (P) and blood vessels (V), indicating endochondral ossification. Longitudinally sectioned tissues were stained with Hematoxylin and Eosin. magnification x 106.

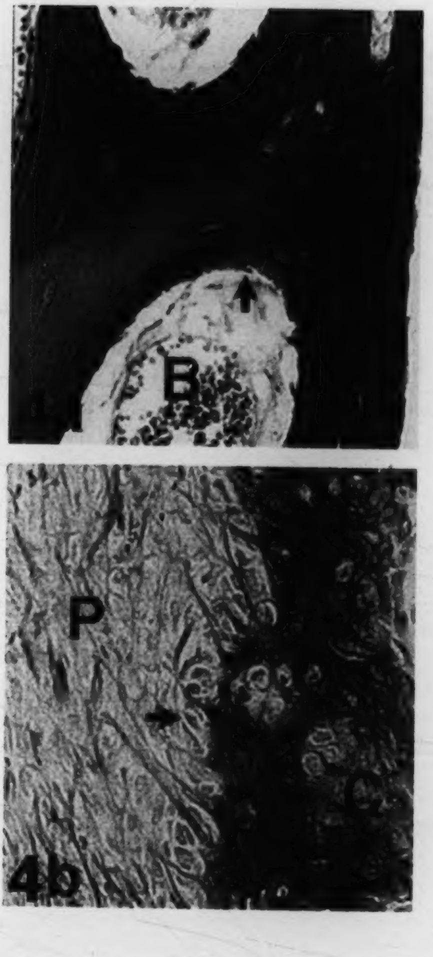

Figure 2.4. Bony structure in base section. a: Bony trabeculae of spongiosa lined by osteoid (arrow). B: bone marrow. b: Cortical bony structure (C) covered with periosteum (P). Small arrow points osteocytes. In the periosteum (P), mesenchymal cells differentiate into bone forming cells, osteoblasts (large arrow). Because of many similarities in staining patterns between the base and middle sections, the micrograph of the latter is not shown. Longitudinally sectioned tissues were stained with Hematoxylin and Eosin. magnification x 330.

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Chapter 3.

Chemical Composition of Antlers from Wapiti (*Cervus elaphus*)[1]

3.1. Introduction

Velvet antlers have been an important traditional oriental medicine for many centuries. With increasing demand of velvet antler, deer farming is now becoming a viable alternative form of animal agriculture in many parts of the world including North America, Europe and New Zealand (Sim 1987). World wide, about five million deer are farmed, most of which serve this demand. Each year over 4,000 tonnes[1] of antler enter international markets to supplement local production in Eastern Pacific Rim Countries. Its use as a food supplement is rapidly growing in Europe and North America. The international market demands antlers of specific size, shape and mineralization.

Antlers grow in length by endochondral ossification, and in diameter by intramembranous ossification (Banks 1974). They are suggested to be a useful model for the study of bone growth and metabolism (Goss 1983). Wapiti antlers are removed at about 60 days of growth (June in Northern photoperiods) when the main beam just begins to bulb at the 4th tine. Antlers can be divided into four sections, tip, upper, middle and base which are used differently to treat diseases such as anemia, arthritis, hypercholesterolemia, and cancer or to promote health as traditional oriental tonics (Fennessy 1991).

For food researchers, antlers with various biological activities could be a useful component to develop a new food product which can modulate physiology of our body and protect us against diseases. Such a function of food is important in addition to those contributing to nutrition and palatability.

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[1] *A version of this chapter has been published. HH Sunwoo, T Nakano, RJ Hudson and JS Sim 1995. J. Agri. Food Chem. 43: 2846-2849.*

Despite high demand, limited information is available concerning chemical composition of antler (Sim and Hudson 1991; Sim et al. 1995). Such information is important to understand the growth and development of antlers as well as their function as nutritional supplement or pharmaceutical agents. The present study was undertaken to determine protein, amino acid, carbohydrate and fatty acid contents in the four sections of wapiti antler.

3.2. Materials and Methods

Preparation of Antlers

Velvet antlers were obtained from four 4 year old wapiti stags averaging 365 kg maintained at the University of Alberta's Ministik Research Station. These animals were kept on a high quality pelleted diet with 17.9% crude protein, 0.84% calcium and 0.44% phosphorus provided *ad libitum*. Velvet antlers (approximately 65 days after casting of the buttons from the previous set) were harvested from each stag under local or general anesthesia by a veterinarian, and each antler was divided with a butcher knife into four sections (tip, upper, middle, and base) of the main beam (see Chapter 1, Figure 2.1.). Sample of each section was skinned, weighed and homogenized with a meat grinder. Because antler growth depends on endochondral ossification and intramembranous ossification (Banks 1974), the velvet skin, which is not the tissue where ossification takes place, was not included in the present analysis. This was thought to provide analytical data more specific to the growing antler tissue than those with unskinned antler. However, we are planning to analyze velvet skin in the near future. Homogenized samples were freeze-dried and stored at -20°C until analyzed. Care of animals and procedures used were in accordance with the guidelines of the Canadian Council on Animal Care (1993).

Chemical Analyses

Antler samples were analyzed for protein, ash, calcium, phosphorus and magnesium by the methods of Association of Official Analytical Chemists (1984). For amino acid analysis, samples were hydrolyzed with 6 N HCl at 110°C for 24 h. Hydroxyproline content in the hydrolysate was determined by the method of Stegemann and Stalder (1967). The content of collagen was calculated by multiplying the content of hydroxyproline by 7. The separation and quantification of amino acids other than hydroxyproline were accomplished by HPLC (Model 5000, Varian Associates, Inc., Sunnyvale, CA) equipped with a Varian fluorochrome detector (Jones and Gilligan 1983). The precolumn derivatization was carried out by mixing the sample with a fluoraldehyde reagent, *o*-phthaldialdehyde, in a 1:1 ratio, using a Varian autosampler which injects the resulting derivative into the column with a delay time of 12 s. The analytical column (4.6 x 150 mm) was packed with a 3-micron, Supelcosil LC-18 (Supelco Canada, Mississauga, Ontario) reversed phase. The guard column (4.6 x 50 mm) was packed with a 20- to 40-micron Supelcosil LC-18 reversed phase. Chromatographic peaks were recorded and integrated using a Shimadzu Ezchrom Chromatography Data System (Shimadzu Scientific Instruments, Inc., Columbia, MD).

Total lipids were determined by extracting samples with chloroform-methanol (2:1 v/v) according to Folch et al. (1957). The lipid samples were dried under nitrogen and were methylated using boron trifluoride methanol according to the method of Metcalfe and Pelka (1961). The fatty acid methyl esters of total lipids were analyzed by a varian (Model 3700) gas liquid chromatography using an SP2330 capillary column (30 m x 0.5 mm, inside diameter). The initial column temperature was 120°C for 1 min. The temperature was programmed to increase by 5°C per min until 190°C. The final temperature of 190°C was held for 5 min. The gas-flow rates were: nitrogen, 34 ml/min; hydrogen, 30 ml/min; and air, 300 ml/min. Chromatogram peaks were integrated by using the same equipment as for the amino-acid analysis.

Uronic acid contents were determined by the carbazole reaction (Kosakai and Yoshizawa 1979) after digesting samples with twice crystallized papain (Sigma) (Scott 1960). Samples of sections 2 to 4, which were found to contain bony tissues of varying amounts were decalcified using 10% disodium EDTA containing 0.05 M Tris, pH 7.4 before papain proteolysis. The digests were also assayed for sulfated glycosaminoglycan (GAG) by the dimethylmethylene blue dye binding method (Farndale et al. 1982). Sialic acid was determined by the method of Warren (1959) after hydrolysis of samples in 0.1 N sulfuric acid at 80°C for 1 h. The chromophore formed was extracted using 1-propanol (Nakano et al. 1994) instead of cyclohexanone used by Warren (1959).

Statistical Analyses

One-way ANOVA was used to determine the difference among means of antler weight, dry matter, contents of protein, collagen, uronic acid, glycosaminoglycan, sialic acid, lipid, ash, calcium, phosphorus, and magnesium, and amino acid and fatty acid profiles. When significant differences occurred ($p < 0.05$), Scheffe's F test was used to determine the difference between means (Steel and Torrie 1980). All analyses were conducted using version 4.01 of StatView (Abacus Concepts, Inc., Berkeley, CA 94701). Data were presented as means ± SEM.

3.3. Results

Results of analysis of each section of skinned antlers are summarized in Table 3.1. The weights of antlers were highest ($p < 0.05$) at the upper section, higher ($p < 0.05$) in the middle than in the base, and lowest ($p < 0,05$) at the tip section. The contents of dry matter, collagen, calcium, phosphorus and magnesium were lowest ($p < 0.05$) in the tip section, and increased downward (upper < middle < base). The proportion of collagen in total protein showed a similar changing pattern. In contrast, the contents of protein and lipid were highest ($p < 0.05$) in the tip, and decreased downward with no difference in the lipid value between the middle and the base sections. Uronic acid contents were higher ($p < 0.05$) in the tip and the upper sections than in the middle and base sections. Sulfated GAG contents decreased ($p < 0.05$) downward with no difference between the middle and the base sections. Sialic acid contents were highest ($p < 0.05$) in the tip section and decreased with no difference between the upper and the middle sections.

The proportion of individual amino acids (Table 3.2.) did not reflect the section related variations of total protein with the exception of lowest ($p < 0.05$) arginine content in the base section and highest ($p < 0.05$) contents of tyrosine and isoleucine in the tip section. The variations in the contents of glycine and alanine, the amino acids present in relatively large amounts in collagen were, however similar to those of collagen.

The fatty acid profiles of the four sections are shown in Table 3.3. Oleic acids (C18:1ω7 and 9) accounted for more than half of total fatty acids. The proportions of C16:0, C16:1ω7, and C20:2ω6 decreased ($p < 0.05$), while those of C18:0, C18:2ω6, C20:4ω6, C22:4ω6 and ω-6 increased ($p < 0.05$) downward. The fatty acid C18:3ω6 (0.16%) was found only in the tip section. The proportions of the remaining fatty acids (C14:0, C18:3ω3, C20:1ω9, C20:3ω6, C22:6ω3, SAFA, MUFA, ω-3 and ω-6) and the ω-6 to ω-3 ratio were similar among the four sections.

3.4. Discussion

There is little information concerning chemical composition of wapiti antlers. However, limited number of studies have been published on antlers of other cervids. Ullrey (1983) in a study of whole velvet antlers from white-tailed deer reported that dry matter accounted for 20% of wet weight, and protein and ash, 80 and 20% of dry matter, respectively. In the present study, values calculated for the skinned whole antler were 31% for dry matter, and 57 and 39% for protein and ash, respectively. The apparent differences between the results of the two studies may be smaller if corrections were made for antlers having skin in the present data. The skin was not included in the present analysis (see Materials and Methods for explanation). Scott and Hughes (1981) reported that hydroxyproline and uronic acid concentrations in deer antler were 42 mg/g and 300 mg/g, respectively. The hydroxyproline value may be close to that (31.4 mg/g) calculated for skin free whole antler in this study assuming that the sample analyzed by Scott and Hughes (1981) had skin. Their uronic acid value was much lower than ours (7.4 mg/g after correction). Miller et al. (1985) reported that calcium, phosphorus, and magnesium concentrations in the unskinned middle section of all-white-tailed-deer antlers were 190.1, 101.3, and 10.94 mg/g dry weight, respectively. The samples analyzed by Miller et al. (1985) appear to be ossified to a greater extent than those analyzed in the present study.

Collagen has been suggested to be the major protein in the antler (Goss 1983). However, little is known about the distribution of this protein in the antler. The results indicated that the amount of collagen in dry tissue increases downward (approximately 1.4, 2.5 and 3.2 times higher in the upper, middle, and base sections, respectively than in the tip section) with concomitant increase in mineral contents. The proportion of collagen of total protein also showed a similar trend. The major function of collagen is to provide mechanical strength to the tissue. It appears that proximal tissue (tissue closer to the center of the body), which sustains distal tissue, requires more collagen than does distal tissue, and that collagen is involved as organic element to reinforce mineralized tissue structure.

The growth center of antler resembles the growth plate (physis) of long bone in that longitudinal growth depends on endochondral ossification (Banks 1974). The proximal portion of tip section and the distal portion of upper section is the sites where endochondral ossification takes place. It has been proposed that anionic molecules of GAG chondroitin sulfate in the growth plate has important roles as ion-exchangers in endochondral bone formation (Hunter 1987). Our recent study showed that chondroitin sulfate is the major GAG in the four sections of wapiti antler. Thus, the greater concentrations of chondroitin sulfate uronic acid or dimethylmethylene blue dye reactive GAG observed in the tip and the upper sections than in the remaining sections are not surprising. In addition to collagen and GAG contents, those of lipid, sialic acid, tyrosine, isoleucine and C18:3ω6 fatty acid were also highest in the tip section. The biological importance of these compounds is unknown.

Antlers have been used for many centuries as an important medicine and tonic in oriental countries such as China, Japan and Korea. However, not much is known about the elements of antler which have beneficial effects on physiological functions. Zhao et al. (1992) reported presence of complement-activating proteoglycan containing chondroitin sulfates in antlers of *Cervus nippon* Temmick. Chondroitin sulfate may be a potentially important carbohydrate in the antler as a food. It has been reported that oral administration of chondroitin sulfate resulted in reduction of pain in osteoarthritis patients (Gross 1983 cited by Paroli et al. 1991). We have recently found increased hematocrit values in chickens fed wapiti antler powder. Unpublished data from this laboratory also showed that intraperitoneal administration of wapiti antler extract to mice resulted in increased growth rate in the animals suggesting presence of growth factors in the extract used. Further investigation of molecules with growth promoting activity in wapiti antlers is under progress in our laboratory.

The present study provides previously unreported information of overall chemical composition of developing wapiti antler. Quantitative variations were observed in proteins, carbohydrates, minerals and lipids among the sections. These variations appeared to be related to the difference in function among sections. However, no satisfactory explanation of the role of each element is available at present. The present analysis also provides a basis for the study of wapiti antler composition in relation to animal age, nutrition, environment, and drying method which should facilitate efficient production of high quality of antler as a food as well as pharmaceutical agents.

Table 3.1. Weights and chemical analysis of velvet antler

	Antler Sections			
Items	Tip	Upper	Middle	Base
Weight(g)	38.37 ± 3.65^{a}	834.32 ± 43.73^{b}	656.48 ± 27.31^{c}	427.23 ± 11.97^{d}
Dry Matter(%)	14.36 ± 0.19^{a}	24.95 ± 0.86^{b}	31.35 ± 0.49^{c}	42.05 ± 0.66^{d}
Protein(%)	69.08 ± 0.88^{a}	61.50 ± 0.77^{b}	57.13 ± 0.41^{c}	49.27 ± 1.08^{d}
Collagen(%)	10.01 ± 0.52^{a}	14.35 ± 1.38^{b}	25.83 ± 0.84^{c}	31.99 ± 1.26^{d}
Lipid(%)	18.94 ± 1.00^{a}	2.67 ± 0.07^{b}	1.02 ± 0.05^{c}	0.50 ± 0.04^{cd}
Uronic acid(%)	1.24 ± 0.17^{a}	1.36 ± 0.11^{a}	0.16 ± 0.02^{b}	0.11 ± 0.01^{b}
Sulfated GAG(%)	3.73 ± 0.47^{a}	4.67 ± 0.27^{b}	0.34 ± 0.03^{c}	0.26 ± 0.03^{c}
Sialic acid(%)	0.61 ± 0.01^{a}	0.30 ± 0.06^{b}	0.25 ± 0.03^{b}	0.09 ± 0.02^{c}
Ash(%)	9.40 ± 0.27^{a}	34.45 ± 0.61^{b}	39.87 ± 0.60^{c}	48.04 ± 0.40^{d}
Calcium(%)	0.42 ± 0.01^{a}	3.32 ± 0.07^{b}	11.77 ± 0.23^{c}	16.50 ± 0.36^{d}
Phosphorus(%)	0.39 ± 0.02^{a}	1.84 ± 0.05^{b}	6.95 ± 0.07^{c}	8.59 ± 0.12^{d}
Magnesium(%)	0.04 ± 0.00^{a}	0.08 ± 0.00^{b}	0.19 ± 0.01^{c}	0.29 ± 0.01^{d}

All values for chemical analysis are on a dry matter basis.

$^{a\text{-}d}$ Means in rows with different letters are significantly different ($p < 0.05$).

GAG is the abbreviation of glycosaminoglycan.

Table 3.2. Analysis of amino acid in velvet antler (% of dry matter)

	Antler Sections			
Amino acid	Tip	Upper	Middle	Base
Aspartic acid	6.64 ± 0.23	7.46 ± 0.37	6.76 ± 0.29	6.78 ± 0.24
Glutamic acid	10.52 ± 0.36	9.99 ± 0.39	10.07 ± 0.28	11.20 ± 0.25
Serine	3.04 ± 0.20^{a}	3.73 ± 0.16^{b}	3.29 ± 0.16^{ab}	3.51 ± 0.12^{ab}
Histidine	2.11 ± 0.15^{a}	2.84 ± 0.19^{b}	1.87 ± 0.20^{a}	1.57 ± 0.07^{a}
Glycine	7.63 ± 0.66^{a}	9.70 ± 0.23^{b}	12.23 ± 0.68^{c}	17.42 ± 0.14^{d}
Threonine	3.29 ± 0.15^{ab}	3.62 ± 0.17^{a}	3.07 ± 0.19^{ab}	2.90 ± 0.10^{b}
Arginine	5.15 ± 0.36^{a}	5.51 ± 0.12^{a}	5.97 ± 0.16^{a}	7.26 ± 0.16^{b}
Alanine	5.74 ± 0.25^{a}	6.99 ± 0.21^{b}	7.50 ± 0.21^{b}	8.87 ± 0.20^{c}
Tyrosine	2.11 ± 0.10^{a}	1.92 ± 0.09^{ab}	1.60 ± 0.10^{b}	1.62 ± 0.04^{b}
Valine	4.34 ± 0.15^{ab}	4.75 ± 0.26^{a}	3.92 ± 0.23^{ab}	3.69 ± 0.12^{b}
Phenylalanine	3.34 ± 0.17	3.90 ± 0.23	3.19 ± 0.21	3.18 ± 0.08
Isoleucine	2.37 ± 0.08^{a}	1.77 ± 0.08^{b}	1.69 ± 0.06^{b}	1.76 ± 0.04^{b}
Leucine	6.42 ± 0.29^{ab}	7.22 ± 0.41^{a}	5.67 ± 0.38^{b}	5.11 ± 0.17^{b}
Lysine	3.87 ± 0.24	4.33 ± 0.12	3.79 ± 0.21	3.96 ± 0.13

$^{a-c}$ Means in rows with different letters are significantly different ($p < 0.05$).

Table 3.3. Fatty acid composition (% of total fat) of velvet antler

	Antler Sections			
Fatty acid	Tip	Upper	Middle	Base
C14:0	1.18 ± 0.03	1.15 ± 0.01	0.87 ± 0.04	1.06 ± 0.23
C16:0	16.27 ± 0.66^{a}	15.10 ± 0.12^{a}	14.44 ± 0.44^{ab}	12.19 ± 0.58^{b}
C16:1ω7	4.26 ± 0.35^{a}	2.09 ± 0.03^{b}	1.19 ± 0.07^{bc}	0.83 ± 0.15^{c}
C18:0	7.79 ± 0.21^{a}	7.58 ± 0.18^{a}	9.28 ± 0.09^{b}	9.61 ± 0.54^{b}
C18:1ω7 and 9	55.72 ± 1.69	59.31 ± 0.63	54.81 ± 1.12	57.40 ± 2.54
C18:2ω6	2.58 ± 0.09^{a}	3.22 ± 0.13^{b}	5.20 ± 0.09^{c}	4.61 ± 0.12^{d}
C18:3ω6	0.16 ± 0.00^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
C18:3ω3	0.86 ± 0.20	0.64 ± 0.05	1.02 ± 0.03	0.87 ± 0.05
C20:1ω9	0.19 ± 0.03	0.22 ± 0 .05	0.46 ± 0.10	0.18 ± 0.03
C20:2ω6	1.98 ± 0.15^{a}	1.28 ± 0.04^{b}	0.43 ± 0.02^{c}	0.45 ± 0.02^{c}
C20:3ω6	0.73 ± 0.13	0.79 ± 0 .01	0.80 ± 0.17	0.64 ± 0.05
C20:4ω6	2.99 ± 0.19^{a}	3.40 ± 0.03^{a}	5.28 ± 0.06^{b}	5.62 ± 0.52^{b}
C22:4ω6	0.39 ± 0.02^{a}	0.51 ± 0.12^{a}	0.78 ± 0.03^{b}	0.82 ± 0.09^{b}
C22:6ω3	1.29 ± 0.03	1.30 ± 0.02	1.36 ± 0.03	1.37 ± 0.07
SAFA[e]	27.15 ±0.89	25.79 ± 0.29	26.82 ± 0.53	25.12 ± 1.29
MUFA[f]	61.89 ± 0.93	63.07 ± 0 .23	58.33 ± 0.69	60.50 ± 1.96
ω-3[g]	2.15 ± 0.21	1.94 ± 0.06	2.38 ± 0.05	2.25 ± 0.12
ω-6[h]	8.82 ± 0.24^{a}	9.20 ± 0.21^{a}	12.48 ± 0.33^{b}	12.14 ± 0.58^{b}
ω-6:ω-3	4.19 ± 0.47	4.75 ± 0.07	5.25 ± 0.25	5.41 ± 0.04

[a-d] Means in rows with different letters are significantly different ($p < 0.05$).

[e] Total saturated fatty acids include C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, and C24:0.

[f] Total monounsaturated fatty acids include C15:1ω5, C16:1ω7, C18:1ω7, C18:1ω9, and C20:1ω9.

[g] Total omega-3 fatty acids include C18:3ω3 and C22:6ω3.

[h] Total omega-6 fatty acids include C18:2ω6, C18:3ω6, C20:2ω6, C20:3ω6, C20:4ω6, and C22:4ω6.

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Chapter 4.

Chemical and Pharmacological Characterization of Water Extracts from Antlers of Wapiti (*Cervus elaphus*): Growth Promoting Effect *in vivo* and *in vitro*[1]

4.1. Introduction

Velvet antlers from deer species have been used as traditional oriental medicine for many centuries. The conventional and clinical observations from the Eastern world have convincingly showed that antler extract contains active components which have various effects on body metabolism (as tonic), immune functions, and anti-aging. They also have hypotensive and hypocholesterolemic effects, and enhance gonadotropic functions. However, limited information is available concerning the active substances of antlers.

Interestingly, many studies have been carried out to understand the growth and development of antlers. Antlers are unique growing tissues among mammalian species, in which a cycle of growth, maturation, mineralization, casting and regeneration occurs annually. Antlers grow in length by endochondral ossification, and in diameter by intramembranous ossification (Banks 1974). Antlers are the fastest growing tissues among mammals showing the maximum growth rate of antlers of elk (*Cervus elaphus*) upto 2.75 cm/day (Goss 1970). There is limited information available concerning growth factors involved in the antlerogenesis (Bubenik 1983). Ko et al. (1986) reported the presence of epidermal growth factor in the velvet skin of growing antler of red deer, and partially characterized it. Suttie et al. (1989, 1991) reported close association of insulin growth factor-I with antler growth.

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[1] *Some of the data presented in this chapter have been published. HH Sunwoo, T Nakano and JS Sim 1997. Can. J. Anim. Sci. 77: 343-345.*

According to the demand of the international markets, velvet antlers from wapiti are harvested at approximately 60 days of growth (June in northern photoperiods) after button-casting. The harvested antlers are a growing stage and are composed of different types of tissues including cartilaginous (distal) and osseous (proximal) tissues. The main beam of such unilaterally growing antlers of wapiti can be divided into four sections, tip, upper, middle and base (Sunwoo et al. 1995) according to the cell populations (Chapter 2). The growth of antler is dependent on the proliferation and maturation of chondrocytes that occur in the tip and upper, respectively (Banks and Newbrey 1983). Chemical composition in growing antler of wapiti differs among sections (Sim and Hudson 1991; Sunwoo et al. 1995). The tip section as the growth center of antler contains relatively abundant proteoglycans measured as uronic acid (Sunwoo et al. 1995). Proteoglycans are composed of protein core to which one or more glycosaminoglycan (GAG) chains are covalently attached. Proteoglycans have been suggested to be important molecules involved in cell proliferation and differentiation (Hardingham and Fosang 1992).

Various information must be accumulated to use antlers as nutritional supplements or pharmaceutical agents. Therefore, this report shows our preliminary investigations on the chemical and pharmacological characteristics of antler extracts both *in vivo* and *in vitro*. The objectives were: 1) to monitor growth promoting activity of rats and in bovine skin fibroblasts as affected by the extract from the tip section of growing antler of wapiti both in ; 2) if so, to fractionate the extract using gel chromatography and to examine each fraction for fibroblast growth promoting activity; and 3) to characterize the active substances in the fraction using electrophoresis, enzymatic digestion, and western blotting.

4.2. Materials and Methods

Preparation and Fractionation of Water Soluble Extracts

Fresh samples of velvet antlers were obtained in June at 65 days after button-casting from four 4 year old wapiti stags averaging 365 kg body weight. These animals were kept at the University of Alberta Ministik Research Station. Care of animals and procedures used were in accordance with the guidelines of the Canadian Council on Animal Care (1993).

The sample of tip section, taken from the main beam of each antler was washed in cold water, dissected free of velvet skin and stored at -20°C until extracted. The frozen samples of tip sections of antlers were thawed at 4°C, and homogenized with cold deionized water (5 ml/100 mg sample) to obtain water soluble extracts (WSE). The extracts were centrifuged at 40,000 x g, and 4°C for 30 min, and the supernatant was sterilized through 0.22 μm filters (Millipore Corporation, Bedford, MA.) and used for rat and cell culture studies. The contents of protein in WSE were measured by the method of Lowry (Lowry et al. 1951) using bovine serum albumin (BSA) as a standard.

The WSE was fractionated at 4°C using a 1.0 x 110 cm column of Sephacryl S-300 (Pharmacia Biotech Canada Inc. Baie d'Urfe, PQ) which was equilibrated and eluted with phosphate buffered saline (PBS, 0.15 M NaCl, 0.0027 M KCl, 0.0081 M disodium phosphate, and 0.0015 M monopotassium phosphate, pH 7.2) at a flow rate of 3 ml/h. Blue dextran (Pharmacia Biotech Canada Inc.) and tritiated water were used to determine void volume (V_o) and total volume (V_t) of the column, respectively. The eluates were monitored for absorbance at 280 nm to measure protein contents. Each tube was also subject to the measurement of contents of sulfated glycosaminoglycans (GAG) by the dimethylmethylene blue dye binding method (Farndale et al. 1982), and of uronic acid by the method of Blumenkrantze and Asboe-Hansen (1973) using D-glucuronolactone as a standard. Concentrations of these components were monitored because of the high content of proteoglycan in the tip section (Sunwoo et al. 1995), which may be extracted with water. Appropriate fractions were pooled, filtered through 0.22 μm membrane and used for cell culture study (see below).

Rat Growth

Six 22 day old male rats were used in this study to investigate the effect of WSE from antler and divided into 2 groups. Rats were injected intraperitoneally with 1 ml WSE containing 100 μg protein and 1 ml PBS as control. One more injection was intraperitoneally given at 2 wk after the first injection with the same dose. After 4 weeks of first injection, all rats were weighed.

Cell Culture Conditions

Bovine skin fibroblasts (BSF) were isolated from the fresh skin of young adult cattle, obtained at a local abattoir (Gainers Inc., Edmonton), and were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum (FCS) as described (Freshney 1986). After reaching confluence, the cells were subcultured. Cells at the 7-10 th passage were used for the cell culture experiment. The BSF were seeded at a density of 30,000 cells in 2 ml of DMEM supplemented with 10% FCS in 6-well Costar plastic tissue culture plates (Costar Corporation, Cambridge, MA) under an atmosphere of 5% CO_2 at 37°C. The medium was replaced 24 h after seeding by 2 ml of DMEM with 2% FCS. Preliminary experiments with different levels (0, 2, 5 and 10%) of FCS showed suboptimal cell growth with 2% FCS, which was considered to be appropriate condition to detect the effects of WSE and its fractions on the cell growth. The increase of cell number with 2% FCS was slower than that with 5 or 10% FCS, and reached plateau after 3 days of incubation. In contrast, the cell number continued to increase with 5 and 10% FCS with the rate of increase being higher with the latter. No appreciable growth of BSF was observed without FCS.

After replacement by 2% FCS, cells were incubated with different amounts of WSE in 5% CO_2 at 37°C for 72 h to determine the effect of added WSE on the cell growth. Samples of 1.5 to 6 mg of WSE, measured as protein, in 100 µl of PBS were added to 2 ml of medium. For the experiment to examine the mitogenic effect of fractions obtained from a Sephacryl S-300 column, samples of 20 µg protein in 100 µl PBS were added to 2 ml of medium. In both experiments, cell culture with no added WSE (or its fraction) was used as control. Cell numbers were counted with hemocytometer after trypsinization with 0.25% trypsin for 3 min at 37°C. A *t*-test was used to detect significant difference ($p < 0.05$) between means of cell culture results.

Agarose-Polyacrylamide Gel Electrophoresis and Immunoblotting

Electrophoresis was carried out in 0.6% polyacrylamide in 1% agarose and in Tris buffer, pH 6.8 (Scott and Pearson 1978). Two slabs were generally run at the same time, one for staining with Toluidine blue and the other for immunoblotting with monoclonal antibodies (MAbs) to chondroitin sulfate (CS-56; Avnur and Geiger 1984) and to keratan sulfate (AH12; Nakano et al. 1993).

Electrophoretic transfer to nitrocellulose was accomplished in Tris-borate gel electrophoresis buffer without SDS at 40 volts for 2 h. Nitrocellulose sheets were then soaked in 2% bovine serum albumin (BSA) in phosphate buffered saline (PBS), pH 7.2 for 1 h at room temperature. After washing in PBS (three times for 5 min each time) nitrocellulose sheets were incubated separately with MAbs, CS-56 (1:250 in PBS), AH12 (1:250 in PBS), and 6D6 (1:10 in PBS) for 1 h at room temperature. This was followed by washing in PBS, and incubation for 1 h with rabbit anti-mouse IgM for CS-56 and AH12 MAbs. IgM conjugated with horseradish peroxidase (1:1000 dilution in PBS with 1% BSA; Sigma) was used as secondary antibodies. Color was developed by incubating in 0.05% diaminobenzidine tetra-hydrochloride (Sigma) in PBS containing hydrogen peroxide (0.01% w/v) and cobalt chloride (0.033% w/v) for 5 min. Stained blots were then washed several times in water and then dried.

4.3. Results and Discussion

The water soluble extracts (WSE) were injected into rats and growth of rat was examined. The average body weight was 182 and 149 g for 4 weeks in WSE and PBS groups, respectively (Figure 4.1.).

The bovine skin fibroblasts (BSF) were cultured under different levels (0, 2, 5 and 10%) of fetal calf serum (FCS), and their growth rates were compared. The increase of cell number with 2% FCS was slower than that with 5 or 10% FCS, and reached a plateau after 3 days of incubation. In contrast, cell number continued to increase with 5 and 10% FCS with the rate of increase being higher with the latter. No appreciable growth of BSF was observed without FCS. The 2% FCS, which resulted in suboptimal cell growth was used to examine the effect of WSE on the cell growth .

The response of different concentrations of water soluble extract (WSE) on the growth of BSF is shown in Figure 4.2. The addition of WSE promoted ($p < 0.05$) the growth of BSF either with (2%) or without FCS. The increase of cell number was dose-dependent in either case. The stimulation of cell growth observed without FCS suggests presence of growth promoting factor(s) in the WSE.

The WSE was then fractionated using gel chromatography on Sephacryl S-300 to examine which fraction has growth promoting activity in the cell culture system. The elution pattern for protein, sulfated GAG and uronic acid are shown in Figure 4.3. Five protein peaks, I (K_{av}, 0), II (K_{av}, 0.20), III (K_{av}, 0.33), IV (K_{av}, 0.74), and V (K_{av}, 0.84) accounted for 14.2, 20.1, 45.8, 10.0 and 9.9% of total protein recovered, respectively. Sulfated GAG and uronic acid were found in peak I only, suggesting that the peak contains proteoglycan(s). The protein peak fractions were pooled as indicated in Figure 4.3., and tested for a mitogenic activity in BSF culture. Only the pooled material from peak I, which was referred to as WSE-I, demonstrated growth promoting effect ($p < 0.05$) on BSF in culture with 2% FCS (Figure 4.3.). However, in the FCS free medium, the WSE-I had no effect ($p > 0.05$) on the cell growth (results not shown). One interpretation of these results is that the WSE-I contained unknown material(s) which activated unknown factor(s) in the FCS to stimulate cell growth. If so, the growth promoting factor(s) in the unfractionated WSE (see above) is probably activated by the unknown material(s) from the WSE-I to stimulate cell growth. However, no further investigation was undertaken in this study to examine which peak (out of peaks II-V, Figure 4.3.) contained growth promoting factor(s). Ko et al. (1986) reported the presence of epidermal growth factor (EGF) in the WSE from the red deer antler. These authors chromatographed the extract on a column of Sephadex G-50, and obtained two peaks of EGF detected by radioimmunoassay, one of which eluted at the void volume, and a second was retarded in the column. Since the exclusion limit of Sephadex G-50 is 50 times lower than that of Sephacryl S-300 (supplier's information), it is possible that the size of EGF retarded in the Sephadex G-50 is much smaller than that of peak I fraction excluded from Sephacryl S-300 (Figure 4.4.). It remains to be determined if the peak I fraction from wapiti antler contains EGF and/or growth factor(s) other than EGF.

Immunoblotting analysis of WSE-I using anti-CS (Figure 4.5.B, lane 1) and anti-KS (Figure 4.5.B, lane 3) monoclonal antibodies showed presence of epitopes recognized by these antibodies. This suggests that the WSE-I contains at least two sulfated GAGs, CS and KS. These GAGs were also found in bovine nasal cartilage proteoglycan (Figure 4.5.B, lane 2 and 4), although the staining intensity with CS-56 was much lower in this proteoglycan (Figure 4.5.B, lane 2) than in WSE-1 (Figure 4.5.B, lane 1). Proteoglycans and GAGs are important regulators of differentiation and proliferation of chondrocytes (Goetinck 1982; Hardingham and Fosang 1992; Maurer et al. 1994). Further investigations of the characterization of GAGs in the WSE-I, and of the effect of individual GAG on the growth of BSF in culture are under progress in this laboratory.

The tip section of wapiti antler consists of zones of reserve mesenchymes, prechondroblasts, chondroblasts, and chondrocytes (see Chapter 2). It would be interesting to know which types of cells produce the WSE-I material with fibroblast growth promoting activity. In conclusion, the results demonstrated that the WSE stimulated growth of both rats *in vivo* and BSF in culture with or without FCS. When the WSE was fractionated by gel chromatography on Sephacryl S-300, only the fraction (WSE-I) containing GAGs excluded from the column showed growth promoting effects with 2% FCS. The WSE-I, however, did not stimulate cell growth in the FCS free medium. It was suggested that the GAGs in WSE-I may activate unknown factor(s) in the FCS or the WSE to enhance cell proliferation.

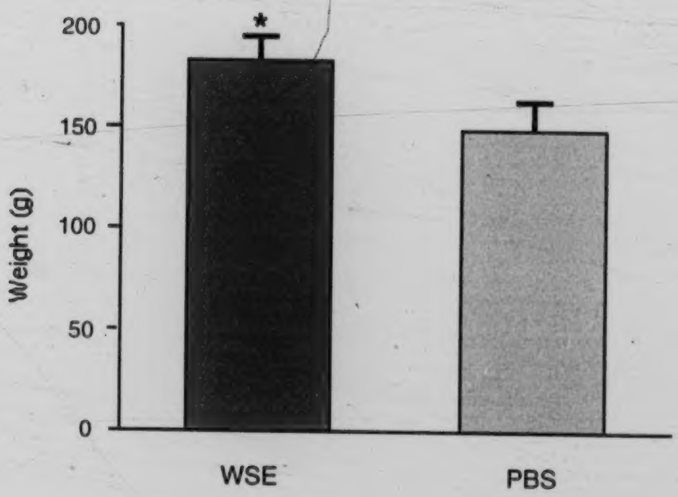

Figure 4.1. Effect of water soluble extract (WSE) of wapiti antler on the growth of rat. The average body weight was measured after 4 weeks injecting water soluble extracts of antler tip and phosphate buffered saline (PBS) as control. Each bar represents the mean (±SEM) obtained from three rats in each group. * Mean greater than that of control group with PBS ($p < 0.05$).

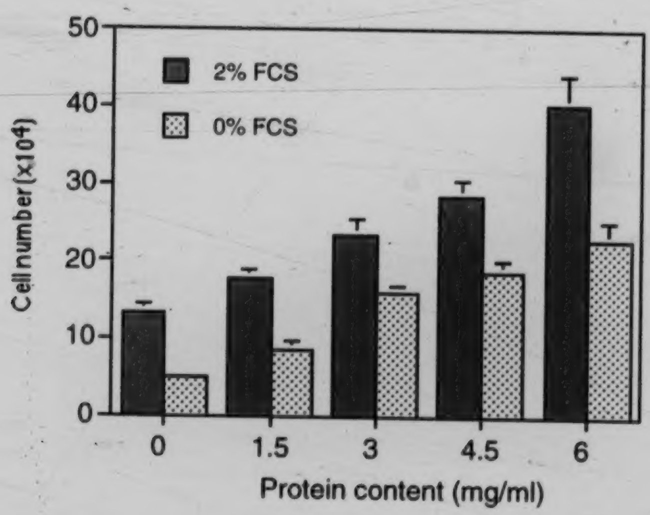

Figure 4.2. Effect of water soluble extract (WSE) on the growth of Bovine skin fibroblasts (BSF). Bovine skin fibroblasts were cultured in triplicate, in the medium containing different amounts of WSE (0, 1.5, 3, 4.5 and 6 mg as protein), and the growth of BSF was examined by counting cell numbers through hematocytometer in 0 and 2% fetal calf serum (FCS) media. Each bar represents the mean (± SEM.) of results from three separate experiments. The increase in cell number was dose dependent ($p < 0.05$).

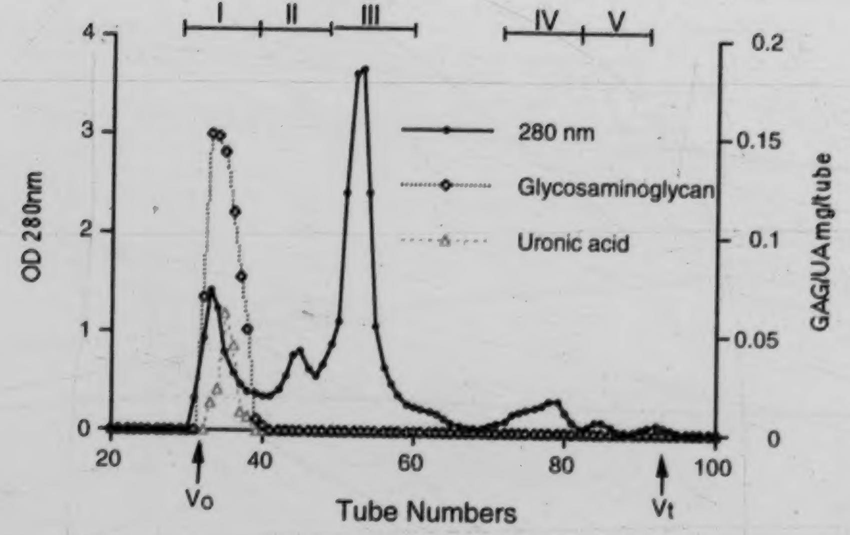

Figure 4.3. Gel chromatography of the WSE. A sample (20 mg) was dissolved in 1 ml of deionized water and applied to a 1 x 110 cm column of Sephacryl S-300, which was eluted with PBS at a flow rate of 3 ml/h. (—) Absorbance at 280 nm (protein). (·······) Results of the dimethylmethylene blue dye binding assay for sulfated glycosaminoglycan. (----) Results of the carbazole reaction for uronic acid. Fractions in each of five protein peaks (I-V) obtained were pooled for further study. V_o and V_t show positions of void volume and total column volume, respectively.

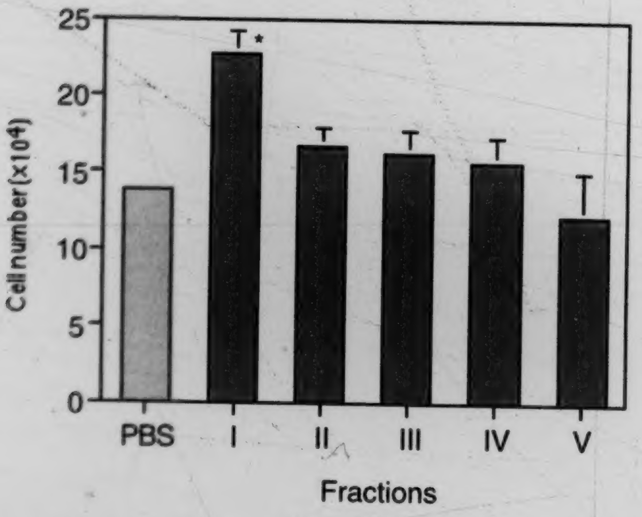

Figure 4.4. Effect of five fractions on the growth of BSF. Bovine skin fibroblasts were cultured, in triplicate, in the medium containing different fractions (I-V) of WSE, and the growth of fibroblasts was examined by counting cell numbers through hematocytometer. Each bar represents the mean (± SEM.) of results from three separate experiments.
* Mean greater than that of control culture with PBS.

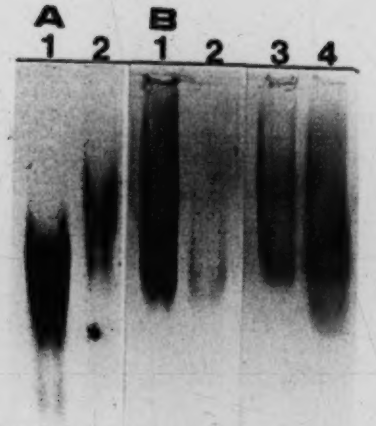

Figure 4.5. Gel electrophoresis (0.6% polyacrylamide) of WSE-I obtained from fractions after Sephacryl S-300 chromatography of WSE in antler. A: lane 1 - standard proteoglycan of bovine nasal cartilage (BNC); lane 2 - WSE-I. Lanes 1 and 2 were stained with Toluidine blue. B: Immunoblots; lanes 1 (WSE-I) and 2 (BNC proteoglycan) are stained with monoclonal antibody to chondroitin sulfate; lane 3 (WSE-I) and 4 (BNC proteoglycan) were stained with monoclonal antibody to keratan sulfate (AH12).

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Chapter 5.

The Effect of Antler Powder on the Growth and Immune Response in Rats

5.1. Introduction

Velvet antler from deer family has been used as Oriental herbal medicine for many centuries in Oriental countries such as China, Japan, Korea and South Asia. In the West, velvet antler with various biological activities could be a useful component to develop a new food product which can modulate body processes and protect us against diseases. Despite potential agricultural by-products, however, limited information is available concerning chemical properties of velvet antlers (Sim and Hudson 1991; Sim et al. 1995; Sunwoo et al. 1995).

From Oriental studies, velvet antlers convincingly possess pharmacological effects which influence body metabolism (Takikawa et al. 1972; Lee 1980), promote immune functions (Ko and Song 1986; Li and Wang 1990), slow the aging process (Chen et al. 1992) involving anti-oxidation (Wang et al. 1988), increase hematopoietic activity (Bae 1976; Song 1970; Kim and Park 1982), enhance gonadotrophic functions and have hypocholesterolemic (Yong 1964) and hypotensive effects (Yudin and Dobryakov 1974). There are *in vitro* experiments for the development of pharmaceutical agents. Extracts of tip in the growing antler accelerate wound healings (Bubenik 1990). Wang et al. (1990) reported that polyamines from antler, consisting of putrescine, spermidine and spermine, stimulate the activity of protein and RNA synthesis in tissue culture. Zhao et al. (1992) reported the presence of anti-complementary activity in the glycoprotein conjugates isolated from antler. Recently water soluble fractions from antler stimulate the growth of bovine skin fibroblasts in culture (Sim et al. 1995; Sunwoo and Sim 1996; Sunwoo et al. 1997).

Such information is important to develop the antler product as nutritional supplements or pharmaceutical agents. Thus, the present study was conducted on the efficacy of velvet antlers by using animal system. The major objective of this study was to determine whether dietary antler powder could enhance growth, decrease the cholesterol level, and affect the immune responses in rats.

5.2. Materials and Methods

Animals and Diets

Male wistar rats, received at 21 days of age, were housed in pairs and fed various experimental diets. Room temperature was maintained between 20 and 22°C, and light provided for 12 hours per day. Housing, handling and killing procedures conformed to policies and recommendations of the University of Alberta Laboratory Animal Care Advisory Committee.

Animals were divided into four groups of 6 rats each in both Ex. 1 and 2. Compositions of experimental diets is shown in Table 5.1. AIN-76 (ICN) synthetic diet which supplied all other nutrient requirements was additionally supplemented with 3% mixture of the gelatin and phosphate-calcium (50:50) in ratio of 80:20, respectively (Control group). Antler powder was fed into 3 groups of 0.5%, 1%, and 3% with replace of a mixture (gelatin and phosphate-calcium as same as control group) of 2.5%, 2% and 0%, respectively.

Velvet antlers from wapiti (*Cervus elaphus*) were obtained from the University of Alberta Ministik Research Station. Care of animals and procedures used was in accordance with the guidelines of the Canadian Council on Animal Care (1993). The antlers were autoclaved for 2 h at 121°C, freeze-dried and ground for feeding rats. The chemical composition of antler powder is shown in Table 5.2.

Experimental Design

Experiment 1 was conducted to obtain information on the effect of antler powder on the growth performance. Body weight, feed intake, organ weights, plasma and liver cholesterol, and total protein and immunoglobulin G (IgG) concentration in serum were determined. During 7 weeks, each of 6 rats from four treatment groups was given a daily allocation of food which increased from 15 g/day during the first week to 20 g/day during the second week and then 24 g, 27 g, 28 g, 29 g and 29 g/day in the third to seventh weeks, respectively.

Experiment 2 was conducted to examine the effect of antler powder on the performance and immune response of rats for 8 weeks. All rats were immunized two times, 1 week apart, with a 1:1 dilution of ovalbumin and Freund's incomplete adjuvant ($0.5\ \mathrm{ml/BW_{kg}}^{0.75}$). The antibody titer was determined 1 and 2 weeks after the last immunization. Body weight, feed intake, organ weights, bone weights, plasma and liver cholesterol, and total protein, IgG concentration and iron in serum were determined for each rat. During 8 weeks each rat from five groups was given a daily allocation of food which was increased from average 16 g/day during the first week to average 19 g during the second week and then average 24 g, 27 g, 27 g and 28 g in the third to sixth weeks, respectively. After immunization, feed intake was reduced to average 22 g/day/rat and 24 g/day/rat in the seventh and eighth weeks, respectively. Feed intake was based on the weight of unconsumed food deducted from the total food offered on a weekly basis.

Analytical Assays

Antler samples were analyzed for protein, ash, calcium, phosphorus, and magnesium by the method of the Association of Official Analytical Chemists (1984). Their amino acids, fatty acids and total lipid were determined as described by Sunwoo et al. (1995). Hydroxyproline content in the hydrolysate was determined by the method of Stegemann and Stalder (1967). Uronic acid contents were determined by the carbazole reaction (Kosakai and Yoshzawa 1979) after digesting samples with twice crystallized papain (Sigma) (Scott 1960). Sialic acid was determined by the method of Warren (1959) after hydrolysis of samples in 0.1 N sulfuric acid at 80°C for 1 h. Total serum protein was determined by the Lowry method (Lowry 1951). Using Sigma kits, plasma total cholesterol, triglycerides, high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) were assayed enzymatically. Lipid was extracted from the liver using the method of Folch et al. (1957). Aliquots of total lipid extracts were taken to determine total cholesterol by the methods described above.

ELISA for Total IgG Concentration and Serum Antibody Titer

The measurement of total IgG was modified from the method of Tirawanchai et al. (1991). Wells of microtiter plates (Costar) were filled with 100 µl of the affinity-purified goat anti-mouse IgG (Sigma) at 10 µg/ml in coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, 0.0031 M NaN_3, pH 9.6). Preliminary experiments disclosed that this coating concentration was in excess of binding capacity. Following antigen adsorption at 4°C for 24 h, the plates were washed with three times 0.5% normal goat serum-phosphate buffered saline (PBS, 0.15 M NaCl, 0.0027 M KCl, 0.0081 M disodium phosphate, and 0.0015 M monopotassium phosphate, pH 7.2)), and wells were filled with 150 µl of 5% normal goat serum-PBS at 37°C for 1 h. After blocking the residual binding sites of the plates, 100 µl of serial 1:2 dilution of heat-inactivated serum (1:125 to 1:16,000) were added in triplicate to the wells. Each plate also contained serial 1:2 dilution of purified mouse IgG standard (Sigma) (10.000 to 0.078 µg/ml in 2.5% normal goat serum-PBS) to prepare the standard curve. Plates were incubated at 37°C for 2 h and washed three times with PBS-Tween (PBST). One hundred µl of a 1:1000 dilution of goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma) in PBST was added as the secondary antibody. After 1 h of incubation at 37°C, the plate was washed with PBST before 100 µl of 1 mg of p-nitrophenyl phosphate (Sigma) was added as a substrate for the enzyme.

The optical density at 414 nm was measured after 1/2 h of incubation at 37°C with ELISA reader and converted to micrograms of IgG per milliliter of serum by using the linear portion of the standard curve.

The ELISA assay was performed with 96-well microtiter plates (Costar). Ovalbumin (200 mg/ml) was dissolved in carbonate-bicarbonate coating buffer, pH 9.6. All other solutions was prepared with a PBST and NaN_3 buffer. Test sera was diluted 1:1000, 1:5000, 1:25000, 1:125000, and 50 µl of each dilution was assayed. Rabbit anti-rat IgG antiserum (Miles) and alkaline phosphates-conjugated goat anti-rabbit IgG antiserum (Miles) was used at a dilution of 1:500. The enzyme substrate consisted of 150 µl p-nitrophenyl phosphate (Sigma), 1 mg/ml, in 10% diethanolamine. Incubation with substrate lasted 15 min, and was stopped by the addition of 50 µl 1.0 N NaOH. The end point of titration was the highest dilution of serum yielding a yellow solution. Results are reported as the reciprocal of this titre.

Spleen and Thymus Examination

For each experiment one animal per group was anesthetized with ether and bled by heart puncture until dead. This procedure was repeated on different days until all the animals were killed. The blood was saved for white blood cell count and differential staining and for determination of serum vitamin A. Using aseptic techniques, the spleen and thymus were removed, weighed and placed in cold RPMI-1640 medium. Portions of the above organs were forced through a #60 mesh screen and the cells suspended in 20 ml RPMI-1640 for 2 min. The cell-containing supernatant was transferred to a second tube, centrifuged at 200 x g and the cell pellet washed twice with RPMI-1640. In the splenic preparations, red cells were sometimes lysed with buffered ammonium chloride. The lymphocytes were finally suspended in culture medium. The cells were counted in a hemocytometer and viability determined by the trypan blue exclusion method.

T- and B-Lymphocyte Mitogen Assay

At the time of killing, the spleen was removed aseptically and prepared individually for assessment of responses to the T-lymphocyte mitogens concanavalin A (Con A; 2 μg/ml) and phytohemagglutinin P (PHA; 0.1 ml, 25 μg of a 1:2 dilution) and the B-lymphocyte mitogen lipopolysaccharide (LPS; 10 μg/ml culture) from E. coli. For thymic lymphoctyes, Con A at 2 μg/ml culture was used. Briefly, single-cell suspensions of 5 million cells/ml were prepared in RPMI-1640 supplemented with 25 mM HEPES buffer, 50 μg/ml gentamicin sulfate, 40 μmol/ml glutamine, and 0.05 μmol/ml 2-mercaptoethanol. Triplicate samples of splenocytes from each animal, with and without mitogens, were placed in 96-well microtiter plates, incubated at 37°C in a humidified incubator containing 5% CO_2 for 24 h. [^{3}H] thymidine (specific activity 2 Ci/mM) was added at 24 h, and cultures were harvested 24 h later onto glass-fiber filter paper discs. Radioactivity was measured with a liquid scintillation counter. The triplicate cpm values were averaged for each animal's response to mitogen or control. These data were then combined with the data from the other animals fed the same diet. Data are presented as the average counts per minute (CPM) ± standard error.

Statistical Analyses

The data were analyzed by analysis of variance and Duncan's multiple range tests (Steel and Torie 1981). All analyses were conducted on a Macintosh computer using version 4.01 of StatView (Abacus Concepts, Berkeley, CA). Data are presented as means ± standard error.

5.3. Results and Discussion

Results of chemical analysis of the antler powder autoclaved from frozen antlers are shown in Table 5.2., 5.3. and 5.4. In the present study, protein and ash as major components of antler powder accounted for 95.8% of dry weight. Lipid, uronic acid and sialic acid were minor components determined (2.74% of dry weight) in that sample. Amino acids (Table 5.3.) of the relatively large amounts in collagen, glycine and alanine, accounted for approximately 24%. The content of gelatin (degraded form of collagen) which can be calculated by multiplying the content of hydroxyproline by 7 accounted for approximately 30% in antler powder. The level of major minerals in antler powder was approximately 21% for calcium and phosphorus. The fatty acid profiles of the antler powder are shown in Table 5.4. Three C16:0, C18:0, and C18ω:1 accounted for more than 50% of total fatty acids. Unknown fatty acids were in large amounts (17.6%). The proportions of monounsaturated fatty acids (18.9%) and polyunsaturated fatty acids (18.9%) were similar in antler powder.

First experimental results on the body performance of growing rats under the normal condition feeding antler powder for 7 weeks showed no significant differences for body and organ weight including liver, testes, spleen and thymus (results not shown). From oriental studies, antler extracts are known to low cholesterol levels in blood and liver (Yong 1964). On the contrary, rats in four groups were not different in plasma levels of cholesterol and HDL-C, LDL-C levels of cholesterol in liver (results not shown).

Beisel (1982) reviewed effects of deficiency of nutrients on antibody production. The deficiency of nutrient decrease not only antibody production, but body weight of rats showing malnutrition. In the present study, only 3% AP group appears to be the decrease of antibody production (3.43 mg/ml) with no significant body weight change (Table 5.5.). This result indicates the existence of unknown factor(s) in antler powder which might influence immune system in rats. However, responses of T- and B-lymphoctyes in spleen to optimal concentrations of the mitogens ConA, PHA and LPS were not statistically different (Table 5.6.).

A second experiment was conducted to examine the immune response of rats immunized intraperitoneally with ovalbumin emulsified with an equal volume of Freund's incomplete adjuvant. Ovalbumin is a monomeric phosphoglycoprotein with a molecular weight of 43 to 45 KDa as a major allergen of egg (Bush and Hefle, 1996). The average initial weights from Control A to 5% AP groups were 80.6 ± 3.7, 81.6 ± 3.3, 81.2 ± 3.6, 81.1 ± 2.7 and 80.6 ± 2.1, respectively. Following 8 weeks, body and liver weights of rats were increased ($p < 0.05$) in groups fed AP (Table 5.7.). The body weight of rats in control group appears to be affected under the immunized condition other than under the normal feeding shown in the first experiment. Liver weights were greatest in AP groups ($p < 0.05$), but the proportion of liver and body weight did not show any significant difference. The proportion of spleen and body weight is greatest in 3% AP group ($p < 0.05$). The weight of right and left femur was not different among groups (results not shown). Feed consumption was not significantly different among groups before and after immunization (results not shown). Plasma total cholesterol, HDL-C, LDL-C were significantly higher in AP groups than in control group. The HDL-C/LDL-C ratio was significantly different among groups (Table 5.8.). These changes appear to reflect the involvement of unknown factor(s) derived from the AP. The increased HDL-C/LDL-C ratio may be important for the prevention of the risk of coronary heart disease.

From serum analyses, total content of protein in serum was not different among groups. Serum antibody titer decreased in all AP groups ($p < 0.05$), while the total IgG contents were significantly decreased ($p < 0.05$) in rat fed 3% antler powder (Table 5.9.). Lymphocytes in spleen and thymus were not different in all groups. As same as the observation of the first experiment, mitogenic responses of T- and B-lymphocyte in spleen were similar among groups fed different levels of antler powder (Table 5.10.). Immunization did not affect the mitogenic responses in the present study. The specific antibody response against ovalbumin tended to be less sensitive in the antler powder fed groups than in the control group. Thus, our data suggest that there are significant factor(s) in the antler powder that enhances the growth performance or affects the immune system of rats.

Since AP lowered humoral immune response by antibody titers measured in the present study, it would seem that the previous report (Ko and Song 1986), the effect of antler extract on the increase of hemagglutinin titers and Rosette forming cells in mice, would not match with our result. Further research to determine the effect of dietary AP on other parameters of immune response is warranted. In the past decades, there has been an increasing interest in the potential of velvet antlers to enhance immune system and decrease cholesterol. Antlers were composed of minerals, amino acids, fatty acids, glycolipids and glycoproteins, or unknown compounds (Fennessy 1991). It has been postulated that a specific component and/or multi-compounds in velvet antlers may possess the activities. Sunwoo et al. (1995) reporting the presence of the collagen, lipid, minerals, sialic acid, and uronic acid.

The present study investigated previously reported information of cholesterol concentrations and immune responses in rats fed antler powder prepared by heat processing. In experiment 1, feeding antler powder diet for 7 weeks showed no significant effect on the growth rate and feed intake as well as concentrations of total cholesterol in liver. High HDL-C/LDL-C ratio in AP groups appear to reflect the prevention of the risk of coronary heart disease. In experiment 2, under immunized conditions, body and liver weights of rats were increased ($p < 0.05$) in AP groups. The total IgG contents were significantly decreased ($p < 0.05$) in rat fed 3% AP. The specific antibody response against ovalbumin showed the tendency of less sensitive in the antler powder fed groups than in the control group. However, no satisfactory explanation of the role of AP is available at present. The present findings also provides a basis for the study of wapiti antler efficacy in relation to active component(s) which expand antler industry for the development of antler products as a food as well as pharmaceutical agents.

Table 5.1. Composition (g/kg) of rat diets (AIN 76A supplemented with 3% mixture of gelatin and phosphate-calcium in ratio of 80:20, respectively)

Components	Control	0.5% AP[a]	1% AP	3% AP
Casein	194	194	194	194
Methionine	2.91	2.91	2.91	2.91
Corn Starch	145.5	145.5	145.5	145.5
Sucrose	485	485	485	485
Cellulose	48.5	48.5	48.5	48.5
Corn oil	48.5	48.5	48.5	48.5
Salt mix	33.95	33.95	33.95	33.95
Vitamin mix	9.7	9.7	9.7	9.7
Choline	1.94	1.94	1.94	1.94
Gelatin	24	20	16	0
AP	0	5	10	30
Ca/P[b]	6	5	4	0
Total	1000	1000	1000	1000

[a] AP, antler powder.

[b] Ca/P, Calcium and phosphorus.

Table 5.2. Nutrient compositions of antler powder

Items	Antler powder
Dry matter	99.4 %
Protein	62.8%
Hydroxyproline	4.20%
Lipid	2.14%
Uronic acid	0.33%
Sialic acid	0.27%
Ash	33.0%
Calcium	14.1%
Phosphorus	6.9%
Potassium	0.25%
Magnesium	0.39%
Sodium	0.80%
Salt	2.05%
Zinc	86 ppm
Manganese	1 ppm
Copper	7 ppm
Iron	422 ppm
Sulfur	0.45 ppm
Metabolizable energy	2.67 MCal/kg

Table 5.3. Amino acids of antler powder (percent of dry matter)

Amino acid	Antler powder (%)
aspartic acid	3.69
glutamic acid	5.84
serine	1.81
histidine	1.53
glycine	15.58
threonine	1.79
arginine	3.88
alanine	8.39
tyrosine	0.77
valine	2.87
phenylalanine	2.03
isoleucine	0.98
leucine	3.38
lysine	2.96

Table 5.4. Lipid classes and fatty acids of antler powder

Lipid Classes	percent (%)	Fatty acid composition	percent (%)
Triglyceride (%)	34.44	C14:0	3.25
Free Fatty Acid (%)	4.66	C15:0	2.22
Cholesterol (%)	2.45	C16:0	23.16
Phospholipid (%)	58.46	C16:1	1.23
		C17:0	1.15
		C18:0	11.85
		C18:1	16.90
		C18:2ω6	7.41
		C18:3ω3	0.65
		C20:0	0.66
		C20:1ω9	0.39
		C20:2ω6	0.28
		C20:3ω6	0.73
		C20:4ω6	6.13
		C20:5ω3	0.74
		C22:0	0.68
		C22:1	0.35
		C22:5ω3	3.01
		SAFA[a]	44.56
		MUFA[b]	18.87
		PUFA[c]	18.94
		ω-3	4.40
		ω-6	14.55
		ω-6: ω-3	3.31
		Unknown	17.63

[a] SAFA, saturated fatty acids.

[b] MUFA, monounsaturated fatty acids.

[c] PUFA, polyunsaturated fatty acids.

Table 5.5. Hematological parameters and total lymphocytes of rats fed antler powder for 7 weeks

Diet	Protein (mg/ml)	IgG[d] (mg/ml)	Spleen Cells x 10^9	Thymus Cells x 10^9
Control[b]	64.96±5.46[a]	4.28±0.29[a]	8.67±0.48[a]	6.45±0.57[a]
0.5% AP[c]	48.63±1.03[a]	4.37±0.40[a]	9.01±0.67[a]	7.43±0.72[a]
1.0% AP	58.87±8.39[a]	4.19±0.25[a]	8.39±0.42[a]	7.98±0.87[a]
3.0% AP	51.17±4.31[a]	3.43±0.29[a]	9.34±0.40[a]	8.40±0.53[a]

[a] Means in columns with different superscripts are significantly different ($p < 0.05$).

[b] Control, AIN 76A supplemented with 3% mixture of gelatin and phosphate-calcium in ratio of 80:20, respectively.

[c] AP, antler powder.

[d] IgG, immunoglobulin G.

Table 5.6. Effect of dietary antler powder on the Con A, PHA, and LPS mitogenic responses of splenocytes[a]

Diet	Normal cpm	Con A cpm	PHA cpm	LPS cpm
Control[b]	2,799±1,009[a]	27,662±10,231[a]	12,269± 3,386[a]	7,172±1,698[a]
0.5% AP[c]	4,009±1,907[a]	33,243± 7,540[a]	11,477± 5,443[a]	7,764±2,736[a]
1.0% AP	3,841±1,180[a]	24,255± 5,778[a]	14,276± 3,711[a]	5,765±2,136[a]
3.0% AP	3,477±1,727[a]	22,866± 6,441[a]	16,119± 4,782[a]	6,551±1,742[a]

[a] Means in columns with different superscripts are significantly different ($p < 0.05$).

[b] Control, AIN 76A supplemented with 3% mixture of gelatin and phosphate-calcium in ratio of 80:20, respectively.

[c] AP, antler powder.

Table 5.7. Body and organ weights of immunized rats fed antler powder for 8 week[a]

Diet	BW	Liver	Liver/BW	Spleen	Spleen/BW	Thymus	Thymus/BW
	g	g	g/100g	g	g/100g	g	g/100g
Control[b]	432±11.9^{a}	16.5±0.6^{a}	3.82±0.06^{a}	1.24±0.08^{a}	0.29±0.01^{ab}	0.82±0.05^{a}	0.19±0.01^{a}
0.5% AP[c]	457± 8.1^{b}	19.2±0.9^{b}	4.18±0.15^{a}	1.16±0.06^{a}	0.25±0.01^{a}	0.79±0.08^{a}	0.17±0.02^{a}
1.0% AP	461± 5.4^{b}	19.2±0.5^{b}	4.17±0.11^{a}	1.17±0.12^{a}	0.25±0.03^{a}	0.78±0.08^{a}	0.17±0.02^{a}
3.0% AP	453±10.4^{b}	18.8±0.9^{b}	4.09±0.15^{a}	1.42±0.10^{a}	0.31±0.02^{b}	0.85±0.04^{a}	0.19±0.01^{a}

[a] Means in columns with different superscripts are significantly different ($p < 0.05$).

[b] Control, AIN 76A supplemented with 3% mixture of gelatin and phosphate-calcium in ratio of 80:20, respectively.

[c] AP, antler powder.

Table 5.8. Lipid status of immunized rats fed antler powder for 8 week[a]

Diet	Plasma Lipid %	TG mmol/L	Cholesterol mmol/L	HDL-C	LDL-C	HDL-C/ LDL-C
Control[b]	0.33±0.04^{a}	1.58±0.09^{a}	2.17±0.09^{a}	1.54±0.09^{a}	0.33±0.01^{a}	4.66±0.11^{a}
0.5% AP[c]	0.41±0.04^{a}	1.67±0.12^{a}	2.43±0.18^{a}	1.84±0.10^{b}	0.40±0.01^{b}	4.62±0.13^{a}
1.0% AP	0.40±0.01^{a}	1.39±0.10^{a}	2.94±0.11^{b}	2.17±0.07^{c}	0.43±0.02^{b}	5.09±0.27^{ab}
3.0% AP	0.48±0.05^{a}	1.35±0.11^{a}	3.12±0.09^{b}	2.36±0.09^{c}	0.45±0.02^{b}	5.30±0.07^{b}

[a] Means in columns with different superscripts are significantly different ($p < 0.05$).

[b] Control, AIN 76A supplemented with 3% mixture of gelatin and phosphate-calcium in ratio of 80:20, respectively.

[c] AP, antler powder.

Table 5.9. Hematological parameters and total lymphocytes of immunized rats fed antler powder for 8 week[a]

	Protein (mg/ml)	IgG[d] (mg/ml)	Titer ABS	Spleen No.	Thymus No.
Control[b]	52.06 ± 3.84^{a}	4.83 ± 0.17^{a}	2.36 ± 0.11^{a}	9.42 ± 0.66^{a}	9.26 ± 0.36^{a}
0.5% AP[c]	53.26 ± 3.91^{a}	4.94 ± 0.30^{a}	1.27 ± 0.18^{b}	10.70 ± 0.72^{ab}	11.33 ± 1.14^{a}
1.0% AP	48.59 ± 2.29^{a}	4.66 ± 0.30^{a}	1.02 ± 0.08^{b}	12.50 ± 1.33^{b}	11.94 ± 0.93^{a}
3.0% AP	52.32 ± 2.95^{a}	3.43 ± 0.26^{b}	1.27 ± 0.06^{b}	12.87 ± 1.26^{b}	10.80 ± 1.30^{a}

[a] Means in columns with different superscripts are significantly different ($p < 0.05$).

[b] Control, AIN 76A supplemented with 3% mixture of gelatin and phosphate-calcium in ratio of 80:20, respectively.

[c] AP, antler powder.

[d] IgG, immunoglobulin G.

Table 5.10. Effect of dietary antler powder on the Con A, PHA, and LPS mitogenic responses of splenocytes in immunized rats[a]

Diet	Normal cpm	Con A cpm	PHA cpm	LPS cpm
Control[b]	1,300±700[a]	12,268±1,386[a]	18,238± 4,121[a]	2,005± 900[a]
0.5% AP[c]	909±176[a]	14,474±1,727[a]	12,823± 3,517[a]	1,840± 632[a]
1.0% AP	1,029±563[a]	14,265± 4,050[a]	18,886± 5,776[a]	1,681± 736[a]
3.0% AP	1,159±360[a]	14,257± 3,314[a]	14,473± 2,333[a]	2,327±1,149[a]

[a] Means in columns with different superscripts are significantly different ($p < 0.05$).

[b] Control, AIN 76A supplemented with 3% mixture of gelatin and phosphate-calcium in ratio of 80:20, respectively.

[c] AP, antler powder.

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Chapter 6.

Glycosaminoglycans from Growing Antlers of Wapiti (*Cervus elaphus*)[1]

6.1. Introduction

Antlers are fast growing protuberances on the skull of male cervids (Goss 1983). Antlers grow in length by endochondral ossification, and in diameter by intramembranous ossification (Banks 1974). Thus, growing antlers are composed of different types of tissues including cartilaginous and osseous tissues as seen in long bones containing growth plate (physis).

Antlers contain collagen as a major protein with relatively small amounts of glycosaminoglycans (GAGs) and glycoproteins (Sunwoo et al. 1995). With the exception hyaluronic acid which exists as a free polymer, other GAGs (see Appendix) such as chondroitin 4- and 6-sulfates, dermatan sulfate are, keratan sulfate and heparan sulfate, covalently attached to core proteins to form proteoglycans, and have important physiological functions such as water retention and electrolyte control (Hardingham and Fosang 1992). It has been reported that cartilage proteoglycans regulate differentiation and proliferation of chondrocytes (Goetinck 1982). Thus, GAG and proteoglycans appear to have important roles for the development of antler. Frasier et al. (1975) histochemically demonstrated GAG chondroitin sulfate (CS) in the cartilage matrix of the deer antler. Chondroitin sulfate was also chemically analyzed in the pilose antler of *Cervus nippon* (Zhao et al. 1992), and fossilized deer antler (Scott and Hughes 1981). Positive effects of CS as pharmacological agents to treat diseases such as arthritis, atherosclerosis, and cancer have been reported elsewhere (Prudden 1985; Matsushima et al. 1987; Dean et al. 1991). Investigations of pharmacological effects of antler GAGs may be important in this regard. There is limited information available on quantitative analysis of antler GAG(s). There are also few studies on GAG distribution in different parts of the antler.

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The objective of this study was to extract GAGs from skin free growing antlers and characterize these GAGs using electrophoresis, enzymatic digestion and gel chromatography. This information is important to the understanding of both antler development and the chemistry of antlers as potential therapeutic agent or food supplement.

6.2. Materials and Methods

Tissues

Samples of velvet antlers were obtained in June at 60 - 65 days after button-casting from four 4 year old wapiti stags averaging 365 kg body weight under local and general anesthesia by a veterinarian. These animals were kept at the University of Alberta Ministik Research Station, and fed *ad libitum* a diet with 17.9% crude protein, 0.84% calcium and 0.44% phosphorus. Care of animals and procedures used were in accordance with the guidelines of the Canadian Council on Animal Care (1993). The main beam of each harvested antler was skinned and divided into four sections (tip, upper, middle and base) as previously described (Sunwoo et al. 1995). The tip section contained soft cartilaginous tissue with no bony structure. Most of the upper section was also cartilaginous with primary spongiosa as a minor tissue. In contrast, bone and bone marrow were major tissues in the middle and base sections. Light microscopic examination with Hematoxylin and Eosin staining showed that the distal end of the tip section consisted of a zone of reserve mesenchymal cells, which was continuous to the zone of prechondroblasts. The remaining portion (proximal portion corresponding to more than half of the tip section) was occupied by a zone of chondrocytes. The upper section was composed of zones of chondrocytes including maturing and calcifying chondrocytes with a relatively small proportion of mineralized tissue. The middle and base sections were composed of trabecular bone structures.

Each section was ground using a meat grinder, and freeze-dried. The freeze-dried samples were defatted using chloroform-methanol (2:1), dried in acetone, weighed and then stored at -20°C until analyzed.

Tissue Decalcification and Digestion with Papain

All dry defatted samples with the exception of samples from tip sections were decalcified using 20% of tetrasodium ethylenediaminetetraacetic acid/0.05 M Tris, pH 7.4. Samples from tip sections and decalcified samples were then digested with twice-crystallized papain (Sigma Chemical Co.) as described (Scott 1960). After proteolysis, cold trichloroacetic acid was added to the digest to a final concentration of 7% (w/v), and the mixture was left at 4°C overnight. Following removal of the precipitated protein by centrifugation, the supernatant was quantitatively transferred to dialysis tubing and dialyzed for 24 h against running tap water and deionized water. After dialysis, the papain digests were freeze-dried, weighed and then stored at 4°C for subsequent analysis.

Analysis of Papain Digests

Uronic acid was determined by the method of Blumenkrantze and Asboe-Hansen (1973) using D-glucuronolactone as a standard. Sulfated GAG content was estimated by the dimethylmethylene blue dye binding method (Farndale et al. 1982). Hydroxyproline was determined by the method of Stegemann and Stalder (1967) after hydrolysis of samples in 6 N HCl at 110°C for 20 h. Sialic acid content was determined by the periodate-thiobarbituric acid reaction (Warren 1959) after hydrolysis of tissues in 0.1 N sulfuric acid at 80°C for 1 h. The chromophore formed was extracted using 1-propanol (Nakano et al. 1994) instead of cyclohexanone used in the original method (Warren 1959). Protein was determined by the method of Lowry et al. (Lowry et al. 1951) using bovine serum albumin as a standard. A *t*-test was used to detect significant difference ($p < 0.05$) between means of analytical results.

Enzymatic Digestion of Glycosaminoglycans

Digestion with *Streptomyces* hyaluronidase (Calbiochem-Nova Biochem) was carried out as described (Brandt et al. 1976). The hyaluronidase cleaves N-acetylglucosamidic linkages in hyaluronic acid (Kresse and Glössl 1987). Digestions with chondroitinase-ABC and chondroitinase-ACI (both obtained from Seikagaku America) were carried out following the procedures of Saito et al. (1968). Chondroitinase-ABC cleaves ß-1, 4-N-acetylhexosamidic linkage to either D-glucuronic acid or L-iduronic acid, whereas chondroitinase-ACI cleaves ß-1, 4-N-acetylhexosamidic linkage to D-glucuronic acid (Kresse and Glössl 1987). All enzymes were tested beforehand for activity against samples of appropriate GAGs: human umbilical cord hyaluronic acid (Sigma Chemical Co.), whale cartilage CS, shark cartilage CS and hog skin dermatan sulfate (DS) (all obtained from Miles Laboratories).

Electrophoresis

Glycosaminoglycans in papain digests were precipitated with three volumes of 95% ethanol containing 1% (w/v) anhydrous sodium acetate. The precipitate was then washed three times with 75% ethanol, dried, dissolved in water, and electrophoresed on cellulose acetate strips (Gelman Sciences) in 0.1 M pyridine/1.2 M acetic acid, pH 3.5 (Habuchi et al. 1973), and in 0.1 M potassium phosphate, pH 7.0 (Hata and Nagai 1972). The strips were stained with 0.1% (w/v) Alcian Blue 8GX in 0.1% acetic acid, cleared in Sepra Clear II (Gelman Sciences), and subjected to densitometric scanning at 600 nm. The proportion of hyaluronic acid in total GAG was determined by comparing the peak areas for hyaluronic acid and sulfated GAG bands, respectively. The value for hyaluronic acid peak was corrected for the low staining intensity (0.55) (H.H. Sunwoo and T. Nakano, unpublished results) of this non-sulfated GAG relative to whale cartilage CS as 1.0. Digestibility by enzyme was estimated by comparing peak areas of bands from samples incubated with and without enzyme. Samples of GAG were also electrophoresed on 12% polyacrylamide gels containing 0.1% sodium dodecyl sulfate in Tris-borate buffer, pH 8.6 (Mechanic 1979).

Gel Chromatography

A 1 x 110 cm column of Sephacryl S-300 (Pharmacia Biotech Canada Inc.) was equilibrated and eluted with 0.5 M sodium acetate containing 0.02% sodium azide, pH 5.8. Blue dextran and tritiated water were used to determine void volume (V_o) and total volume (V_t) of the column, respectively. The partition coefficient (K_{av}) of GAG was calculated from the formula: $K_{av} = V_e - V_o / V_t - V_o$. Fractions (1 ml) collected were analyzed for sulfated GAG using the dimethylmethylene blue dye binding method (Farndale et al. 1982).

6.3. Results

The yield of papain digest was highest ($p < 0.05$) in the upper section containing mature hypertrophied chondrocytes as major tissues and preosseous tissues as minor tissues where endochondral ossification occurs, and higher ($p < 0.05$) in the tip section consisting of precartilaginous tissues, immature chondrocytes than in the middle and base sections involving ossification (Table 6.1). There were considerable variations in the chemical composition of papain digest between sections of antler (Table 6.1.). The most prominent changes were the decrease in uronic acid concentration (> 2 fold) and the increase in hydroxyproline concentration (> 2 fold) from the distal portion (tip) to the proximal portion (base) of main beam in the antler.

Cellulose acetate electrophoresis of GAG obtained from papain digest by precipitation with ethanol showed two bands for each section of antler (Figure 6.1.). The mobility of a minor slow moving band was identical to that of standard hyaluronic acid. It was digested with *Streptomyces* hyaluronidase, an enzyme specific to hyaluronic acid (Ohya and Kaneko 1970), indicating that the band contained hyaluronic acid. The proportions of hyaluronic acid in total GAG were lower ($p < 0.05$) in the tip (10%) and upper (9%) sections than in the middle (22%) and base (19%) sections. The patterns of major fast moving band were also different between sections. The bands from the tip (Figure 6.1.a) and upper (Figure 6.1.b) sections had their mobilities between positions of standard CS and DS. The bands from the middle (Figure 6.1.c) and base (Figure 6.1.d) sections were much broader than those from the tip and upper sections.

Treatment of papain digests with either chondroitinase-ABC or chondroitinase-ACI resulted in disappearance of all bands with the exception of a slow migrating portion of non-hyaluronic acid band from the middle or the base section, which was resistant to both enzymes (results not shown) accounting for 12% of Alcian Blue stained material. This suggests that the majority of non-hyaluronic acid GAG is CS, and that the proportion of CS in total GAG is higher in the tip and upper than in the middle and base sections.

The results of gel electrophoresis of antler GAG fractions are shown in Figure 6.2. The tip and upper sections had single but broad GAG bands with a mobility slightly faster than that of whale CS. The band was slightly broader in the upper than in the tip section. In contrast, the middle and base sections both had two broad bands with mobilities similar to those of shark and whale CS's, respectively. These results were consistent with those obtained by gel chromatography on Sephacryl S-300 (Figure 6.3.). The tip and upper sections had peaks of K_{av}, 0.15, while the middle and base sections had broader peaks, in that an average 53% of recovered GAGs was eluted at the void volume, and the remaining GAGs were left on the column. The GAG containing fractions were pooled to obtain a single Fraction (tubes 40-55) in the tip and upper sections (Figure 6.3.a, 6.3.b), and two Fractions, I (tubes 31-40) and II (tubes 41-66) in the middle (Figure 6.3.c) and base (Figure 6.3.d) sections. These fractions were examined using electrophoresis on cellulose acetate with and without chondroitinase-ACI treatment.

Electrophoresis patterns of the pooled fractions in pyridine-acetic acid were similar to those of the GAGs from papain digests in both the tip and upper section (results not shown). On the other hand, three bands were seen in Fraction I from either the middle (Figure 6.4.a) or the base (Figure 6.4.b) section. The slowest faint band was identified as hyaluronic acid from its mobility. The second band had its mobility much slower than that of DS, and the third band had highest intensity of Alcian Blue staining with its mobility slightly slower than that of CS. The ratio of GAG in the second band to that in the third band was greater in the middle than in the base section. Fraction II from either the middle or the base section had a broad band with the mobility of its fast migrating front similar to that of standard CS (Figure 6.4.c, 6.4.d). In addition, Fraction II had one broad band, which migrated more slowly than did standard hyaluronic acid. Electrophoresis in 0.1 M potassium phosphate showed similar differences in GAG profiles between sections. The exception was found in Fraction I from either the middle or the base section, in that the mobility of the one band with higher intensity of Alcian Blue staining had its mobility similar to that of standard CS and DS but the second band moved faster than did the standard GAGs (Figure 6.4.e, 6.4.f). These results were not anticipated because in the pyridine-acetic acid system, no band migrated faster than the standard CS (Figure 6.4.a, 6.4.b). Further investigation of this difference was not carried out in this experiment.

Digestion with chondroitinase-ACI resulted in disappearance of bands in all pooled GAG Fractions from Sephacryl S-300 column (results not shown) with the exception of Fraction I from the middle section and Fraction II from the middle or the base section. These Fractions had enzyme resistant materials, which were found as either a fast migrating band (accounting for average 18% of total GAG of Fraction I, Figure 6.5.a) or slow migrating bands (average 22 and 39% of total GAGs of Fractions II from the middle and base sections, respectively, Figure 6.5.b).

6.4. Discussion

The previous analyses of wapiti antler showed increases in tissue concentrations of collagen and minerals (e.g. calcium and phosphorus) and decreases in those of GAG and sialic acid (a constituent sugar of glycoprotein) with increasing distance from the distal end (tip) to the proximal end (base) (Sunwoo et al. 1995). Variations in the yield of papain digest (Table 6.1.) were inversely related to changes in mineral concentrations.

From the proportion of hyaluronic acid and the susceptibility of sulfated GAG to chondroitinase-ACI, we estimate that approximately 10 and 90% of total GAGs are hyaluronic acid and CS, respectively in the tip and the upper sections. In the middle and the base sections, 20, 65 and 15% of total GAGs are hyaluronic acid, CS and chondroitinase-ACI resistant materials, respectively. The high proportion of CS in the antler GAG may not be surprising because the antlers analyzed consisted of cartilage, bone and bone marrow tissues, in which CS is the major GAG (Fisher et al. 1983; Okayama et al. 1988; Nakano and Sim 1994). Concentration of individual GAG can also be calculated from the proportion of GAG (see above), the concentration of uronic acid (Table 6.1), and the yield of papain digest (Table 6.1.). In each section (tip, upper, middle and base), the concentration of hyaluronic acid measured as uronic acid is 0.6, 0.7, 0.3, and 0.2 μg/mg dry-defatted tissue, respectively, and that of CS uronic acid is 5.9, 6.5, 0.9, and 0.6 μg/mg, respectively. The reason for the difference observed in the molecular size of CS between the non-mineralized and mineralized tissues of antler (Figure 6.2. and 6.3.) is unknown.

In conclusion, antler tissues contained CS as a major GAG with small amount of hyaluronic acid in all sections. The middle and base sections also contained chondroitinase-ACI resistant material reactive with Alcian Blue as a minor fraction. The composition of CS determined by cellulose acetate electrophoresis, enzymatic digestion and gel chromatography differed largely between the two parts of antler, the cartilaginous antler containing the tip and upper sections and the bony antler containing the middle and base sections. These findings provide a basis for more detailed investigation of the biochemical composition of GAGs (e.g. dermatan sulfate, keratan sulfate and heparan sulfate) other than CS, hyaluronic acid and proteoglycans from different parts of wapiti antlers.

Table 6.1. Composition of papain digests from growing antler (mean ± SD)

	Sections			
	Tip	Upper	Middle	Base
Yield of papain digest[d]	21.1 ± 2.4[a]	25.5 ± 0.9[b]	8.5 ± 1.1[c]	7.0 ± 0.4[c]
Uronic acid[e]	30.7 ± 1.6[a]	28.0 ± 0.6[b]	16.8 ± 1.3[c]	12.2 ± 0.4[d]
Sialic acid[e]	32.7 ± 2.3[a]	32.8 ± 2.7[a]	30.5 ± 0.8[a]	24.9 ± 1.2[b]
Hydroxyproline[e]	0.9 ± 0.1[a]	1.0 ± 0.1[a]	1.8 ± 0.4[b]	2.2 ± 0.1[b]
Protein[e]	449 ± 22[a]	462 ± 38[ab]	561 ± 52[b]	514 ± 62[ab]

[a-c] Means in rows with different superscripts are significantly ($p < 0.05$) different.

[d] % of dry-defatted tissue.

[e] µg/mg of dry weight of papain digest.

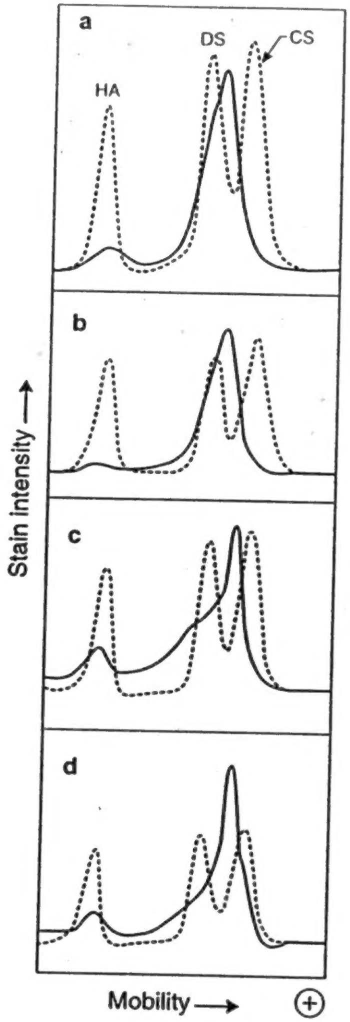

Figure 6.1. Cellulose acetate electrophoresis of antler and standard GAGs in pyridine-acetic acid. Glycosaminoglycan fractions were precipitated by adding four volumes of sodium acetate saturated ethanol to the aqueous solution of papain digest (1 mg/ml). The precipitate was washed three times with 75% ethanol, dried and dissolved in water for electrophoresis. Diagrams show densitometric scans of electrophoretograms of GAG Fractions (______) and standard GAGs (---------), CS, DS and hyaluronic acid (HA) from tip (a), upper (b), middle (c) and base (d) sections of antler.

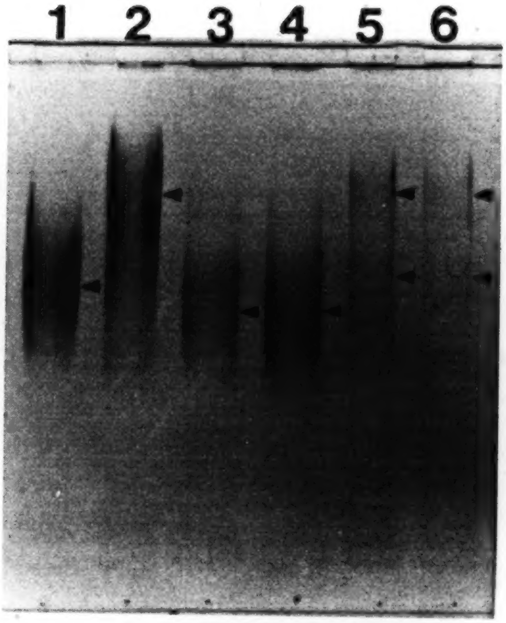

Figure 6.2. Gel electrophoresis (12% polyacrylamide) of antler and standard GAGs. Lanes 1 and 2, standard GAGs, whale CS and shark CS, respectively. Lanes 3-6, GAGs from the tip, upper, middle and base sections, respectively.

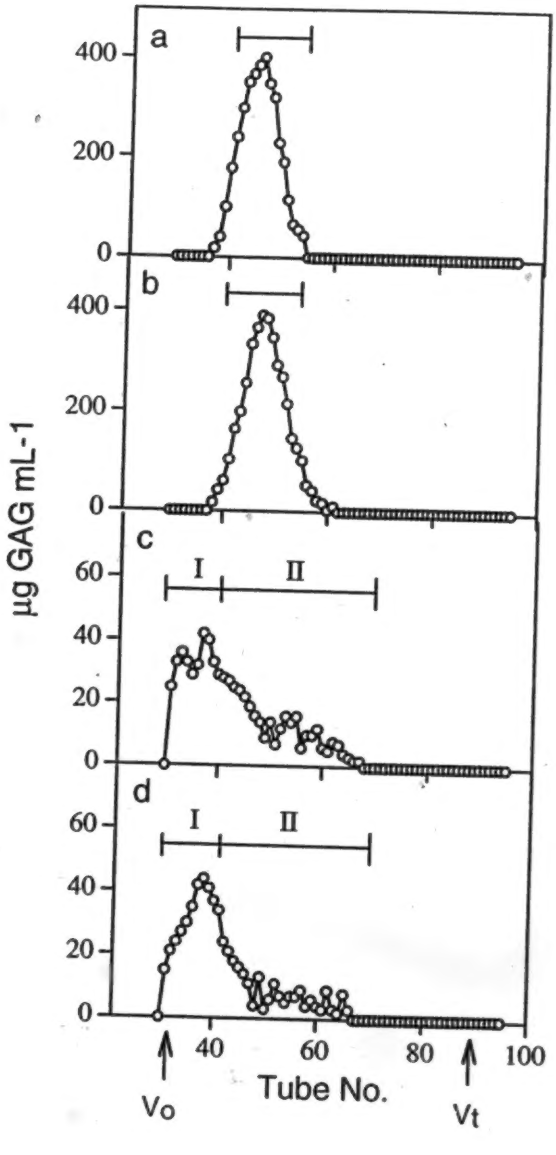

Figure 6.3. Sephacryl S-300 chromatograms of antler GAGs. Elution patterns of GAGs from the tip (a), upper (b), middle (c) and base (d) sections of antler are shown. Bars denote Fractions pooled for further study. V_o and V_t show positions of void and total column volumes, respectively.

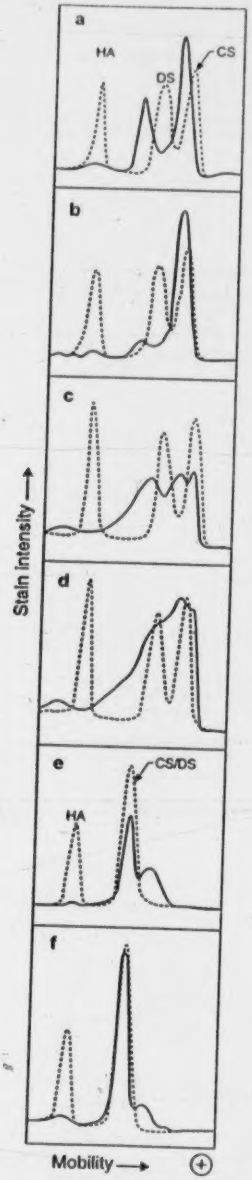

Figure 6.4. Cellulose acetate electrophoresis of Sephacryl S-300 column Fractions. a) and b): Fraction I from the middle and base sections, respectively in pyridine-acetic acid. c) and d): Fraction II from the middle and base sections, respectively in pyridine acetic acid. e) and f): Fraction I from the middle and base sections, respectively in 0.1 M potassium phosphate. (______) GAGs from Fractions I and II. (---------) Standard GAGs, CS, DS and hyaluronic acid (HA).

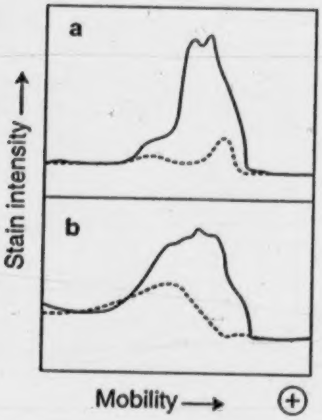

Figure 6.5. Cellulose acetate electrophoresis of Sephacryl S-300 column Fractions incubated with and without chondroitinase-ACI. a) Fraction I from the middle section. b) Fraction II from the base section. (---------) Incubated with enzyme. (_______) Incubated without enzyme (control). Electrophoresis was in pyridine-acetic acid.

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Chapter 7.

Isolation, Characterization, and Localization of Glycosaminoglycans in Growing Antlers of Wapiti (*Cervus elaphus*)[1]

7.1. Introduction

Antlers are the fastest growing tissues among mammalian species (Goss 1983). Unlike horns, they grow and shed every year with a cycle of regeneration, differentiation, mineralization and casting off. The proliferation and differentiation of mesenchymes in distal tip of antlers leads to the development of chondrocytes which are eventually replaced by osseous tissues. The longitudinal growth of antler depends on the endochondral ossification and on the intramembranous ossification in diameter. (Banks 1974). The whole process of antler growth and development including mineralization takes approximately 4 months.

Glycosaminoglycans (GAGs) are acidic polysaccharides composed of repeating disaccharide units of amino sugar (D-glucosamine or D-galactosamine) and uronic acid (D-glucuronic acid or L-iduronic acid) or galactose (in keratan sulfate, KS). With the exception of the nonsulfated GAG, hyaluronic acid, which exists as a free polymer, all GAGs are sulfated and covalently attached to core proteins to form proteoglycans (Jackson et al. 1991). The sulfated GAGs hold water in extracellular matrix as well as interact with many other molecules such as fibronectin, collagen, laminin and growth factors (Hardingham and Fosang 1992). They also play an important role as cation exchangers in the endochondral ossification by promoting hydroxyapatite formation in bones (Hunter 1991).

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Study on GAGs in growing antlers has been limited to histological and partial chemical assay. Histochemical analyses revealed that the extracellular matrix in rapid growing antlers contain sulfated glycosaminoglycans (GAG) (Wislocki et al. 1947), including hyaluronic acid and chondroitin sulfate (CS) (Frasier et al. 1975) which are responsible for calcification of chondrocytes (Mollelo et al. 1963). Quantitative chemical analyses of growing antler of wapiti (*Cervus elaphus*) demonstrated that collagen and sulfated GAGs are the major constituents in both the cartilaginous and bony portions of the structure (Sunwoo et al. 1995). However, little information concerning about the biochemical characterization of GAGs in growing antlers is available.

Thus, the objective of this study was carried out to investigate the composition of GAGs in the cartilaginous and osseous tissues in the main beam of growing antlers from wapiti (*Cervus elaphus*) by using biochemical methods. Glycosaminoglycans were also localized in antler tissues using histochemical and immunohistochemical techniques. This information will provide a better understanding of antler growth which is a unique opportunity to study aspects of cartilage differentiation and bone formation.

7.2. Materials and Methods

Tissues

Antlers were obtained in June (northern photoperiods) at 60-65 days after casting the previous set of hard antlers from four 4-year-old wapiti (*Cervus elaphus*) stags from the University of Alberta Ministik Research Station. Care of animals and procedures used were in accordance with the guidelines of the Canadian Council on Animal Care (1993).

Samples for histochemical and immunohistochemical studies were longitudinally taken from the four sections (tip, upper, middle and base) of the main beam of antlers as previously described (Sunwoo et al. 1995). The distal tip sections contained pre-cartilaginous tissues with no bony structure. The majority of upper section was also of cartilaginous structure with primary spongiosa as minor tissues. In contrast, bones and bone marrow were the major tissues found in the middle and base sections. The collected samples were fixed in 4% buffered formalin, pH 7.3 containing 0.5% cetylpyridinium chloride (Williams and Jackson 1956). The remaining tissues were stored at -20°C until analyzed.

Isolation and Fractionation of GAGs

Frozen antlers were thawed at 4°C, divided into the four sections (Figure 2.1.). A sample of each section was skinned, freeze-dried, and defatted using chloroform-methanol (2:1). All dry-defatted samples were decalcified in 0.5 M tetrasodium EDTA containing 0.05 M Tris and 0.02% sodium azide, pH 7.4 for 2 days at 4°C and then digested with twice-crystallized papain (Sigma Chemical Co.) (4 µg/mg of tissue). After proteolysis, cold trichloroacetic acid was added to the digest to a final concentration of 7% (w/v), and the mixture was left at 4°C overnight. Following removal of the precipitated protein by centrifugation, the supernatant was dialyzed, freeze-dried, and stored at 4°C for subsequent analysis.

Approximately 1 g of papain digest from each section was dissolved in 20 ml of 7 M urea buffer containing 0.05 M Tris and 0.15 M NaCl, pH 6.5, and applied to a column of DEAE-Sephacel equilibrated with the same buffer. Materials containing GAGs were eluted with a linear gradient of 0.15 - 0.8 M NaCl in the above buffer. Fractions were monitored for uronic acid and sulfated GAG. Major fractions showing peaks of uronic acid were collected, dialyzed and then freeze-dried.

The freeze-dried fractions were further fractionated by selective precipitation with varying final concentrations of ethanol in 5 % (w/v) calcium acetate and 0.5 M acetic acid (Meyer et al. 1956). Five fractions (I to V) were collected at ethanol concentrations of 18, 25, 40, 50 and 75 %, respectively.

Analytical Methods

Uronic acid was determined by the carbazole reaction (Kosakai and Yoshizawa 1979) using D-glucuronolactone as a standard. Sulfated GAG content was estimated by the dimethylmethylene blue (DMB) dye binding method (Farndale et al. 1982). Chondroitin sulfate A from whale cartilage (Miles Laboratories) was used as a standard GAG.

Cellulose Acetate Electrophoresis

Electrophoresis of GAGs on cellulose acetate strips (Gelman Sciences) was carried out in 0.1 M pyridine/1.2 M acetic acid, pH 3.5 (Habuchi et al. 1973), 0.1 N HCl (Wessler 1971) and in 0.1 M potassium phosphate, pH 7.0 (Hata and Nagai 1972). The mobility of GAG band in 0.1 N HCl reflects the content of sulfate groups (Wessler 1971). The strips were stained in 0.1% (w/v) Alcian Blue 8GX in 0.1% acetic acid containing 0.02% sodium azide for 3 min, washed in 0.1% acetic acid, cleared in Sepra Clear II (Gelman Science), and then scanned at 600 nm on a Gilford-252 spectrophotometer fitted with a linear transporter.

Enzymatic Digestion of GAG Fractions

Digestion with *Streptomyces* hyaluronidase (Calbiochem-Novabiochem. Co., San Diego) was carried out as previously described (Brandt et al. 1976). Digestions with chondroitinase-ABC and ACI (both obtained from Seikagaku America Inc. Rockville MD, U.S.A.) were carried out by the procedure of Saito et al. (1968). Chondroitinase ABC cleaves the ß-1,4-*N*-acetylhexosamidic linkage to either D-glucuronic acid or L-iduronic acid, whereas chondroitinase ACI cleaves the ß-1,4-*N*-acetylhexosamidic linkage to D-glucuronic acid (Kresse and Glössl 1987). The digests were electrophoresed on cellulose acetate (see above), and the digestibility was estimated by comparing peak areas obtained after scanning electrophoretograms for samples incubated with and without enzyme. The proportion of unsaturated disaccharides released after chondroitinase-ABC digestion was determined using thin layer chromatography as described (Shimada et al. 1987).

All enzymes used were tested beforehand for activity against standard samples of the appropriate GAGs: human umbilical cord hyaluronic acid (Sigma Chemical Co.), whale cartilage chondroitin sulfate A, shark cartilage chondroitin sulfate C (both from Miles Laboratories), and hog skin dermatan sulfate (ICN Biomedicals).

Nitrous Acid Degradation

Aliquots of Fraction I obtained by precipitation with 18% ethanol were incubated with 18% sodium nitrite at room temperature for 80 min (Kraemer 1971). The incubation mixtures were then lyophilized and examined by cellulose acetate electrophoresis.

Gel Chromatography

Samples of GAG fractions containing approximately 100 µg uronic acid were applied to a 1 x 110 cm column of Sephacryl S-300 equilibrated and eluted with 0.5 M sodium acetate containing 0.02% sodium azide, pH 5.8. Fractions (1 ml) collected were assayed for sulfated GAG using dimethylmethylene blue dye binding method (Farndale et al. 1982). The partition coefficient (K_{av}) of GAG was calculated from the following formula: $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_e represents the volume of the peak fraction. V_o (void volume) and V_t (total column volume) were determined using blue dextran (Pharmacia Biotech Canada Inc., Baie d'Urfe, PQ, Canada) and tritiated water, respectively.

Thin Layer Chromatography of Chondroitinase-ABC Digests

A sample of GAGs in each ethanol fraction was digested with chondroitinase-ABC as described (Shimada et al. 1987). Each digestion mixture was chromatographed on thin-layer plates (Silica gel 60F-254 glass plate, 0.25 mm thickness, Merck and Co., obtained through Mandel Scientific Co.) together with standards of unsaturated disaccharides of CS. These standards included 2-acetamido-2-deoxy-3-*O*-(ß-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose (ΔDi-6S), 2-acetamido-2-deoxy-3-*O*-(ß-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose (ΔDi-4S) and 2-acetamido-2-deoxy-3-*O*-(ß-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi-0S), which were obtained from Miles Laboratories. Plates were developed in a solvent system consisting of *n*-propanol-isopropanol-*n*-butanol-water (55:20:5:20, v/v) containing 0.04 M NaCl and 0.01 M ammonia as final concentrations. The proportion of each unsaturated disaccharide was determined as described by Shimada et al. (1987).

Histochemical and immunohistochemical methods

All fixed samples were decalcified in a solution containing 22.5% formic acid and 10% sodium citrate. After dehydration by passage through an ascending alcohol series, the specimens were embedded in paraffin and sectioned at 5 µm. Sections were deparaffinized in xylene, hydrated in a descending alcohol series, and stained with Hematoxylin and Eosin, Alcian Blue and Safranin-O (Drury and Wallington 1967; Rosenberg 1971).

Sections were also stained with monoclonal antibodies (MAbs). The MAbs used were CS-56 (Avnur and Geiger 1984), 6D6 (Pringle et al. 1985) and AH12 (Nakano et al. 1993) recognizing CS, decorin and KS, respectively. Deparrafinized sections were treated with 2% (v/v) hydrogen peroxide in methanol for 30 min, washed three times with phosphate buffered saline (PBS, 0.14 M NaCl, 0.0027 M KCl, 0.0081 M Na_2HPO_4, and 0.0015 M KH_2PO_4) for 5 min in each time. Sections to be incubated with 6D6 and

AH12 were treated with chondroitinase-ABC (0.05 unit/ml of 0.1 M Tris-HCl, pH 8.0) at 37°C for 1 h to digest CS and hyaluronic acid which might otherwise mask tissue antigens. Sections were then soaked in normal rabbit serum (diluted 1 in 20 in PBS) for 30 min and incubated with diluted ascites fluids (1:250 for both CS-56 and AH12) or hybridoma culture supernatant (6D6, 1:10) at room temperature for 1 h. Control sections were incubated with ascites fluid derived from non-immune mouse myeloma cells or myeloma cell culture supernatant. Sections were then washed three times with PBS and incubated with rabbit anti-mouse Ig (G+A+M) (Zymed Laboratories Inc.) diluted 1:50. Final incubation was carried out using mouse-peroxidase-anti-peroxidase complex (Nordic Immunology) diluted 1:300 at room temperature for 30 min. Color was developed with diaminobenzidine tetrahydrochloride (Sigma) and hydrogen peroxide (0.3%).

7.3. Results

The GAG uronic acid recovered in the papain digest, accounted for 0.64, 0.71, 0.14, and 0.09% of dry-defatted tissue in the tip, upper, middle, and base sections, respectively. Elution patterns of papain digests on DEAE-Sephacel were similar among the four sections. A representative chromatogram for the tip section is shown in Figure 7.1. A small proportion of the total uronic acid eluted at 0.3 M NaCl, but most uronic acid eluted at 0.5 to 0.65 M NaCl in all sections of antler. The ratios of minor to major uronic acid peak were: 13:87, 9:91, 14:86 and 12:88 in the tip, upper, middle and base sections, respectively. The 0.3 M NaCl fraction from each section had the electrophoretic mobility identical to that of hyaluronic acid on cellulose acetate and was completely digested with *Streptomyces* hyaluronidase, an enzyme which is specific to hyaluronic acid (Ohya and Kaneko 1970) (results not shown). This minor fraction containing hyaluronic acid was not studied further.

The major GAG fractions eluted from the DEAE-Sephacel column (Figure 7.1.) were further fractionated by differential precipitation with ethanol. The majority of GAGs were recovered in Fractions III and IV, accounting for 90, 91, 83, and 87% of total uronic acid recovered in the tip, upper, middle, and base sections, respectively (Table 7.1.). The remaining GAGs averaging 6, 11 and 4% of total uronic acid were recovered in Fractions I, II and V, respectively. The proportion of uronic acid was higher in Fraction IV than in Fraction III in the tip and upper sections, but lower in Fraction IV than in Fraction III in the middle and base sections.

The ratio of 4-sulfated unsaturated disaccharide to 6-sulfated unsaturated disaccharide (Table 7.1.) tended to be higher in Fractions I-III than in the remaining Fractions. This ratio was, however much higher in the middle and base sections than in the tip and upper sections in Fraction I (approximately 5 times) or III (9 times). In Fractions II-V, the ratio was slightly higher in the upper section than in tip section.

Cellulose acetate electrophoresis in 0.1 M pyridine-1.2 M acetic acid (Figure 7.2.) showed the presence of single bands with the broadness similar to that of standard CS or DS in Fraction I to III. The broadness of band increased in Fractions IV and V. The mobility of band, in general, was slightly slower than that of standard DS in both the Fractions I and II in the four sections of antler with the exception of Fraction I from the tip and upper section which had a band with the mobility of standard DS. The mobility of band increased in the Fraction III or IV, and was between those of standard CS and DS in all sections. In Fraction V, the tip and upper sections had a broad band with its major portion having mobility of standard DS. The middle section had a mobility between those of standard DS and CS. In the base section, the major portion of band had the mobility of standard CS.

Fractions I to V were also examined using cellulose acetate electrophoresis in 0.1 N HCl (Figure 7.3.), in that the mobility of the band reflects the content of the sulfate group. Fractions I to III in all sections had bands with the mobility similar to that of standard CS or DS, indicating that the molar ratio of sulfate to uronic acid or hexosamine is close to 1, as in the CS. The broadness of band increased in Fractions IV and V with average mobility slower than that of the standard CS or DS in all sections.

The results do not reveal whether structures of disaccharide are involved in the copolymer (CS/DS hybrid) or in the homopolymer (CS or DS only). Thus, Fractions I-V were further analyzed using enzymes, chondroitinase ABC and ACI (See Materials and Methods for detail). Average 40 and 49% of Fraction I GAG were found to be susceptible to chondroitinase-ACI and chondroitinase-ABC, respectively in all sections of antler, suggesting that approximately 9% of Fraction I GAG contains DS disaccharides. This accounted for average 0.5% in total GAG recovered in Fractions I to V, and 0.4% in total GAG recovered from the DEAE-Sephacel column. The enzyme resistant materials were unlikely heparan sulfates because the treatment of Fraction I with sodium nitrite showed no appreciable change in its electrophoresis pattern on cellulose acetate. In contrast to Fraction I, Fractions II to V were highly susceptible (100%) to chondroitinase ACI. These results indicated that CS is the major GAG in the four sections of antler.

The major GAG fractions obtained by precipitation with 40 and 50% ethanol (Fractions III and IV, respectively) were further examined using gel chromatography on Sephacryl S-300 (results not shown). The average molecular size of GAG were found to be larger in Fraction III than in Fraction IV in all sections examined (Kav 0.21 vs. 0.32 in the tip and upper sections, and 0.15 vs 0.26 in the remaining sections). The size of GAG was smaller in the tip and upper than in the middle and base sections in either Fraction.

The light microscopic observations of antlers of wapiti (*Cervus elaphus*) were in general similar to those of other cervids (Wislocki et al. 1947; Mollelo et al. 1963; Banks 1974; Price et al. 1996). The distal tip section contained four distinct regions including those of reserve mesenchymes adjacent to the dermis, prechondroblasts, chondroblasts, and chondrocytes. The region of prechondroblasts contained eosinophilic structure (Figure 7.4.a) with low cellularity. These structures were oriented parallel to the long axis of the main beam of antler, and appeared to be precursors of perivascular space. In the upper section, mature hypertrophied chondrocytes adjacent to perivascular spaces were found to lose nuclei staining with Hematoxylin indicating the calcification of chondrocytes (Figure 7.4.b). The observation was also similar to other staining assays. The extracellular matrix surrounding these cells showed a loss of GAG staining with Alcian Blue or Safranin-O (Figure 7.4.c), and was seen to be invaded by osteoblasts, suggesting the occurrence of endochondral ossification in the upper section of antler. The middle and base sections contained bony structures including spongy bone (Figure 7.4.d) with osteoid on its margin and cortical bone.

These observations of different sections in antlers of wapiti in this study were consistent with the report of Banks (1974) suggesting both endochondral ossification and intramembranous ossification. However, we did not find clear evidence for the observations of Rönning et al. (1990) who reported direct conversion of cartilage into bone (by the merge of chondrocytes into bone) without endochondral ossification. The process of endochondral ossification involves several events including growth, maturation and calcification of chondrocytes followed by invasion of bone forming cells, osteoblasts. The intramembranous ossification is dependent on the differentiation of mesenchymal cells into osteoblasts in the cellular periosteum. Osteoblasts form osteoid tissues which are subsequently mineralized to form bone spicules. Compaction of bone spicules develops into cortical bone (Jee 1983).

The intensities of GAG staining with Alcian Blue and Safranin-O (Table 7.2.) were much higher in the tip and upper than in the middle and base sections as expected. However, the staining pattern for GAG differed between these dyes in the middle and base sections (Table 7.2.). Perivascular tissues, spongy bone trabeculae, and cortical bone were stained moderately with Alcian Blue, but were stained little with Safranin-O (Figure 7.4.d).

All control stainings with and without either normal ascites or myeloma cell culture supernatant showed negative results in samples from the four sections of antler (representative negative CS-56 staining result is shown in Figure 7.5.a). The capability of each MAb to immunostain antler tissues is summarized in Table 7.2., and representative stainings of tissues are shown in Figure 7.5. The CS-56 staining was positive in all sections (Figure 7.5.b showing upper section). Particularly, osteoid tended to have stronger CS-56 staining than did the remaining part of spongy and cortical bone (Figure 7.5.c). Positive immunostain with AH12 was observed in cartilage (including both chondrocytes and extracellular matrix) in the tip and upper sections. The overall staining intensity was, however, slightly lower in the tip than in the upper section. The AH12 staining was found to be weak but often positive in the non-cartilaginous tissues including perivascular tissues in the upper section (Figure 7.5.d). No immunostaining was observed in most part of the middle and base sections with the exception of osteoid (Figure 7.5.e), which showed positive AH12 reaction. Positive stainings with 6D6 also were observed in the extracellular matrix of either cartilage or non-cartilage tissues in all sections of the antler. The 6D6 staining was especially intense in the matrix surrounding clustered chondrocytes and often in the area adjacent to calcified chondrocytes (Figure 7.5.f). In the spongy bone, osteoid tended to have stronger immunostain with 6D6 than did the bony trabeculae (Figure 7.5.g). The 6D6 staining was also found to be intense in the cortical bone .

7.4. Discussion

Our results indicate that the chemical structure of CS, accounting for approximately 90% of total uronic acids, is different between cartilaginous and osseous tissues in the antler. This chemical study strongly supports the preliminary data shown in Chapter 6. Among GAGs, hyaluronic acid, DS and KS as minor GAGs were also found in this antler.

The yield of CS liberated from the tissue was estimated from the yield of GAG recovered from the papain digest, the proportion of hyaluronic acid in total GAG recovered from the DEAE-Sephacel column, and the proportions of CS and DS in the ethanol Fractions I to V. The estimated yields were found to be six fold higher in the cartilaginous tissue (average 0.6% of dry-defatted tissue) than in the osseous tissues (0.1%). This is consistent with the results of GAG staining with Alcian Blue and Safranin-O (Table 7.2.).

Most GAGs were recovered as chondroitinase ACI susceptible CS in Fractions III and IV obtained by precipitation with 40 and 50% ethanol, respectively (Table 7.1.). The difference observed in the electrophoretic mobilities between the antler CS and the standard CS from whale cartilage (Figure 7.2.) is probably due to the difference in composition (and thus charge density) between the two samples. This was not investigated further in the present study. A similar trend was observed previously between the whale CS and the chicken CS (Nakano and Sim 1995). It is unknown why the size of CS determined by gel chromatography is larger in Fraction III than in Fraction IV with a broader CS band in the latter on cellulose acetate electrophoresis (Figure 7.2.). Similar differences were previously observed between 40 and 50% ethanol fractions of porcine skeletal muscle epimysial galactosaminoglycans (Nakano et al. 1996a). The broader electrophoretic band in Fraction IV suggests greater variations in the CS chain composition (e.g. sulfate content) in this Fraction than in Fraction III.

The size of CS in osseous tissues is slightly larger than that in cartilaginous tissues of antler. The difference of CS size was observed in bovine bone and cartilage (Sato et al. 1985). A study on CS during the calcification in the rat growth plate showed the content of C-4-S increased from the proliferative zone to the calcifying zone suggesting the importance of GAG sulfation in growth plate calcification (Hagiwara et al. 1995). The calcifying upper section contains slightly more 4-sulfate in all ethanol fractions except Fraction I than the distal tip section with no bony structure does (Table 7.1.). The results indicate that the composition of CS changes as chondrocytes mature and calcify in antler. The greater sulfation of GAG in the upper section reveals an important role in endochondral ossification.

We have recently extracted proteoglycans from the tip and upper sections of wapiti antler, and found that the major proteoglycan was large molecular weight CS proteoglycan excluded from the Sepharose CL-4B (H. H. Sunwoo and T. Nakano unpublished results). This suggests that the majority of CS found in the tip and upper sections of antler are involved in the structure of large CS proteoglycan, aggrecan from hyaline cartilage. This large CS proteoglycan with abundant anionic hydrophilic CS chains (accounting for approximately 90% of dry weight) is thought to provide compressive strength of the load bearing cartilage (Hardingham and Fosang 1992). The CS proteoglycans are also suggested to regulate differentiation and proliferation of chondrocytes (Maurer et al 1994), and endochondral ossification (Hunter 1991), which is a process involved in longitudinal growth of antler (Banks 1974). The abundant CS found in the tip and upper sections of antler, which is apparently subjected to minimum load appear to reflect the importance of CS proteoglycan in both cell growth and endochondral ossification in this fast growing tissue. Recent results of cell culture experiment from this laboratory have shown elevated stimulation of the growth of bovine fibroblast by a CS proteoglycan fraction prepared from the water-soluble extract of antler tip from wapiti (Sunwoo and Sim 1996).

The failure to detect keratan sulfate in the GAG fractions is probably due to insufficient concentration of keratan sulfate present and/or loss of this GAG (Mr. 5000 to 12000, Stuhlsatz et al. 1989) during dialysis. However, keratan sulfate was immunohistochemically detected in all sections of antler. The staining pattern demonstrating lack of this GAG recognized by the MAb AH12 in the mineralized tissues suggests that keratan sulfate, may be more important in non-mineralized tissues including cartilage and osteoid in antler.

Hyaluronic acid, accounting for average 12% of total uronic acid in the antler, was not specifically localized in the present histochemical study. However, by comparing staining patterns with Safranin-O and Alcian Blue, in that the latter has higher affinity to hyaluronic acid than the former (Nakano et al. 1996b), we provide the histochemical evidence that a proportion (but not all) of total hyaluronic acid present are derived from tissues (e.g. perivascular tissue and spongy bone trabeculae) showing positive Alcian Blue staining with little Safranin-O coloration.

The present results suggest the presence of DS in the four sections of antler accounting for average 0.4% of total tissue GAG (see above). This DS was precipitated with 18% ethanol, and resistant to chondroitinase-ACI but susceptible to chondroitinase-ABC. These properties are consistent with those of standard DS from hog skin (Nakano et al. 1996a). It remains to be determined whether the DS chains are composed of DS disaccharides only (forming homopolymers) or of mixtures of DS and CS disaccharides (copolymers). These findings were also consistent with the positive immunostainings of tissues with the MAb 6D6 specific to DS proteoglycans, decorin. However, since 6D6 recognizes the protein core of decorin, it is uncertain whether the DS is involved in the structure of decorin or other proteoglycan(s). Studies of bovine proteoglycans have shown that decorin contains a single chain of DS/CS copolymer in cartilage (Choi et al. 1989), whereas in bone it is substituted with CS chain (Franzén and Heinegård 1984; Scott and Haigh 1985). It may be suggested that the DS found in the cartilaginous portions (tip and upper sections) of antler is derived from decorin. This point must be clarified in the future by isolating decorin from antler tissues and analyzing GAG chains obtained from pure decorin.

Decorin is a low molecular weight proteoglycan (Mr ~100,000), which can bind to collagen fibrils and transforming growth factor-ß (TGF-ß) in vitro (Kresse et al. 1994).

The roles of decorin in vivo is unknown but it has been suggested that decorin may regulate collagen fibrillogenesis (Scott et al. 1981) or to inhibit mineral deposition within the collagen fibrils of non-mineralized tissues (Scott and Haigh 1985). The present findings of the higher intensity of 6D6 staining in the osteoid (non-mineralised tissue) than in the mineralised tissue of spongy bone (Figure 7.5.e) may provide evidence for the latter role. There is little information on the role of decorin in antlerogenesis by controlling the activity of TGF-ß which stimulates collagen (Fine and Goldstein 1987) and proteoglycan (Chen et al. 1987) synthesis.

In conclusion, chondroitin sulfate is a major glycosaminoglycan localized throughout the main beam of wapiti antlers, but its structure is different quantitatively as well as qualitatively between the cartilaginous (tip and upper) and the osseous (middle and base) tissues. Antlers contained, in addition to CS, small amounts of hyaluronic acid, DS, and KS. However, the function of those GAGs with regard to endochondral ossification and bone formation in antler growth remains to be further studied.

Table 7.1. Analysis of the GAG fractions obtained by precipitation with ethanol

	Tip		Upper		Middle		Base	
	III	IV	III	IV	III	IV	III	IV
Uronic acid, %[a]	25.0	64.8	18.3	72.2	50.0	33.3	68.4	18.4
6-sulfated disaccharide, %[b]	43.0	58.0	38.0	53.0	7.0	60.0	7.0	61.0
4-sulfated disaccharide, %[b]	57.0	42.0	62.0	47.0	93.0	40.0	93.0	39.0
4-sulfate/ 6-sulfate	1.3	0.7	1.6	0.9	13.0	0.7	13.0	0.6

Ethanol fractions were shown at 40% (fraction III) and 50% (fraction IV).

[a] % of total uronic acid recovered.

[b] % of total sulfated disaccharide recovered.

Table 7.2. Histochemical and immunohistochemical stainings of growing antlers

Dye or MAb[a]	Tip	Upper	Middle	Base
Alcian Blue	+++	+++	+	+
Safranin-O	+++	+++	-	-
CS-56[b]	+	+	++	++
AH12[c]	++	++	+[e]	+[e]
6D6[d]	++	++	++	++

The staining intensity was graded as - (negative), + (mild), ++ (moderate), and +++ (intense).

[a] MAb, monoclonal antibody.

[b] CS-56, anti-chondroitin sulfate monoclonal antibody.

[c] AH12, anti-keratan sulfate monoclonal antibody.

[d] 6D6, anti-decorin monoclonal antibody.

[e] Positive staining was observed in the osteoid only.

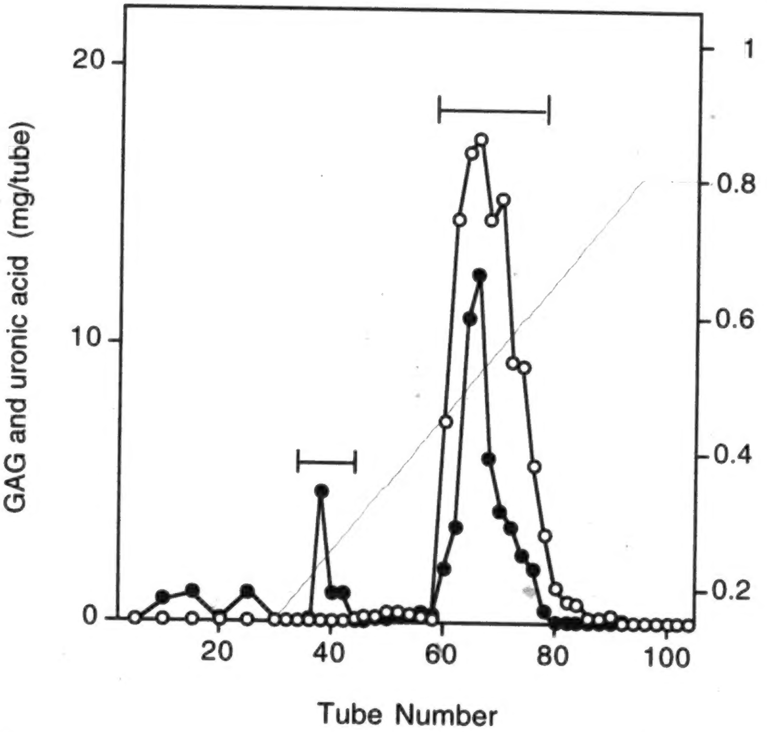


Figure 7.1. Representative DEAE-Sephacel column chromatography of glycosaminoglycans from the antler tissue. The papain digest of the dry-defatted tip section of antler was applied to a 3.5 x 20 cm column of DEAE-Sephacel. Fractions of 9.8 ml were collected and assayed for uronic acid (**o**) and sulfated GAG (o) contents. Bars denote fractions pooled for further study. Elution was performed at room temperature at a flow rate of 27 ml/h.

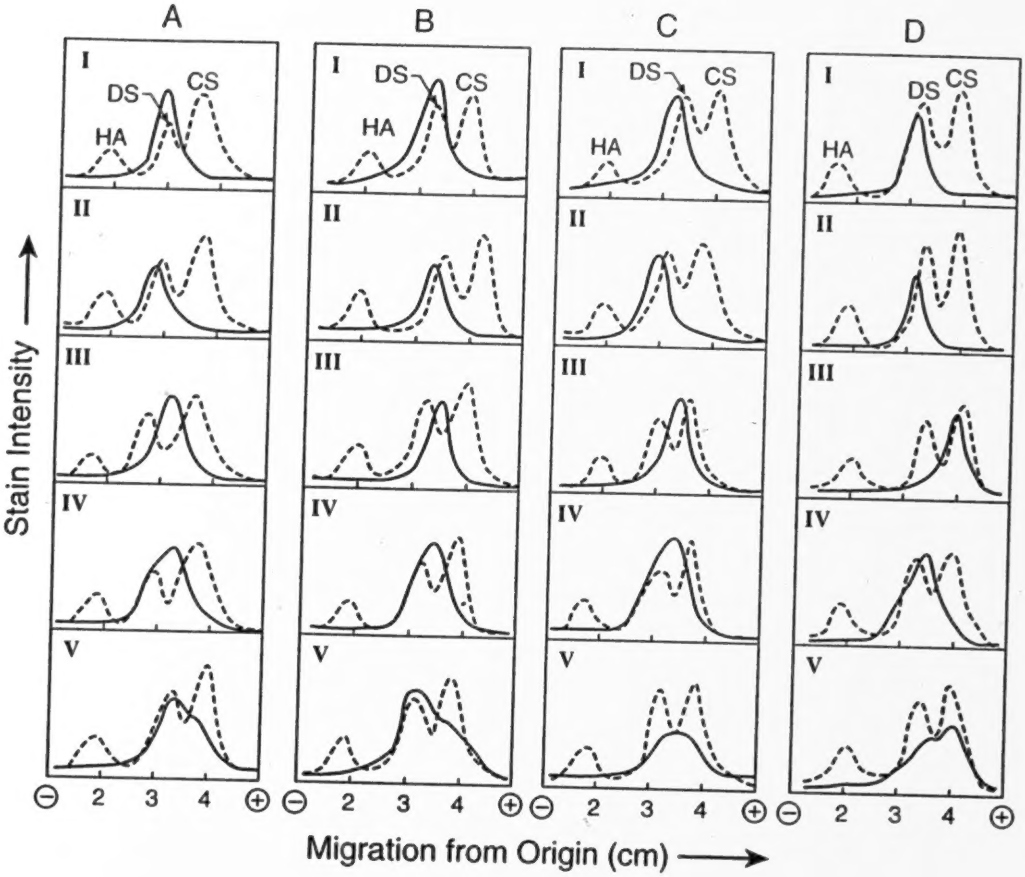


Figure 7.2. Cellulose acetate electrophoresis of GAGs from ethanol Fractions (I to V) in pyridine-acetic acid: A, B, C, and D represent tip, upper, middle and base sections, respectively. (——) GAGs from Fraction I to V, which were obtained by precipitation with different concentrations (18 to 75%) of ethanol. (- - - -) Standard GAGs: HA = hyaluronic acid from human combilical cord; CS = chondroitin sulfate from whale cartilage; DS = dermatan sulfate from hog skin.

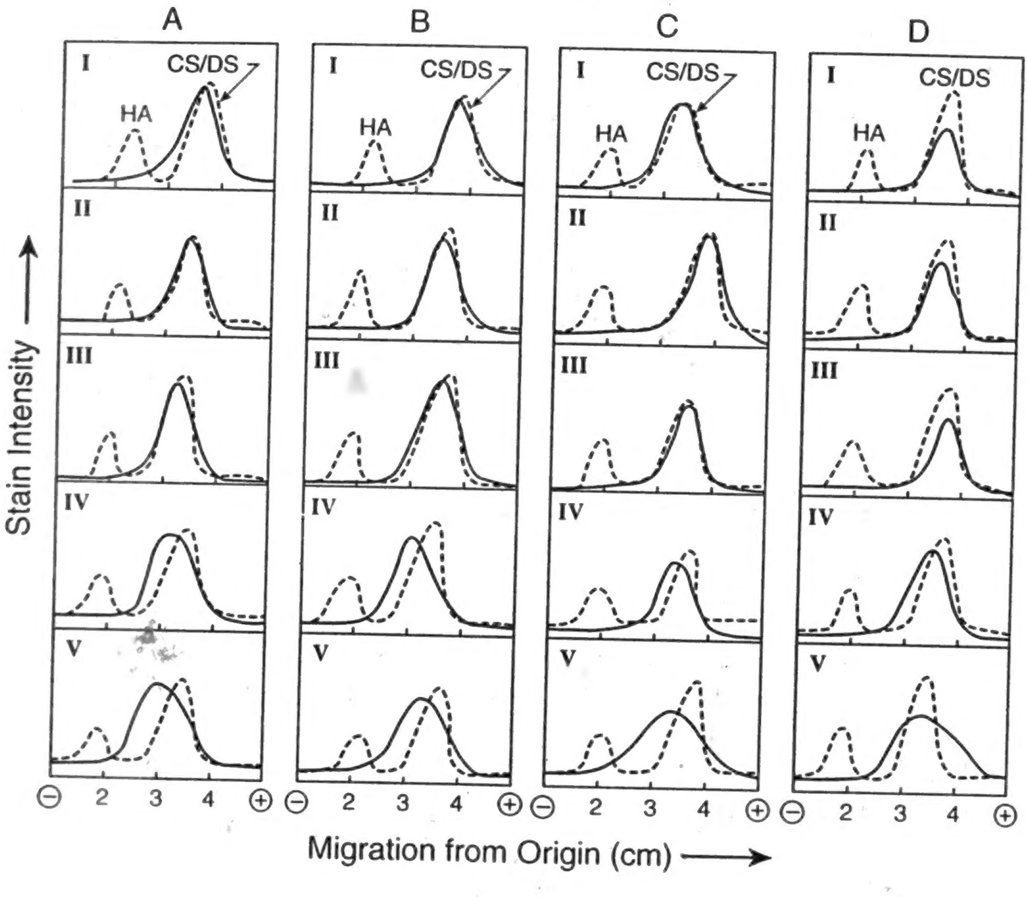


Figure 7.3. Cellulose acetate electrophoresis of GAGs from ethanol fractions (Figure 7.2.) in 0.1 N HCl. Details as in legend to Figure 7.2.

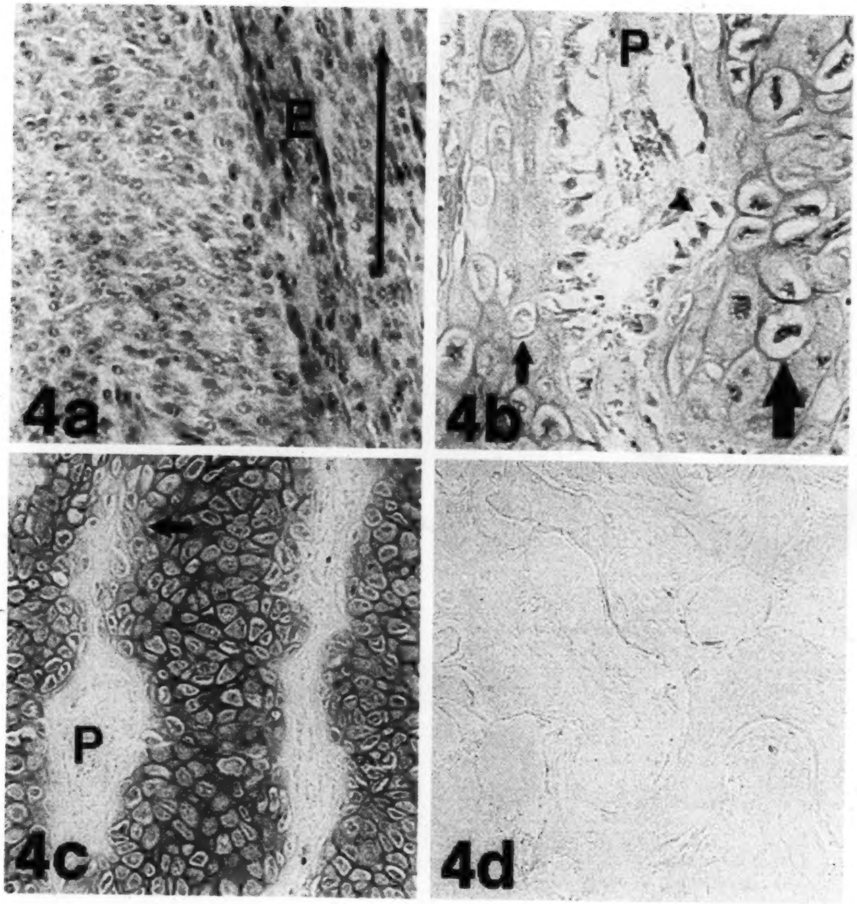
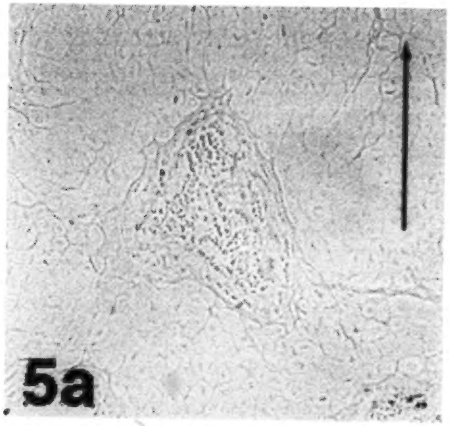


Figure 7.4. Micrographs represent longitudinal sections of growing antlers. Zone of prechondroblasts in distal the tip section (a) stained with Hematoxylin and Eosin. x 260. E: eosinophilic structure. The upper section (b) and (c) showing evidence of endochondral ossification. Mature hypertrophied chondrocytes (large arrow) are calcified, and matrix adjacent to the calcified chondrocytes (small arrow) were invaded by bone forming cells, osteoblasts (arrow head). P: perivascular space. Hematoxylin and Eosin (b) x 260 and Safranin-O (c) x 105. Spongy bone (d) in the base section was negatively stained with Safranin-O. x 105. Long arrow points distal.

Figure 7.5. Immunolocalization of GAGs in longitudinal sections of growing antlers. Immunostaining of mature chondrocytes in upper section (a, b) and bony tissue (c) with the anti-CS MAb CS-56; mature chondrocytes (d) and bony tissue (e) with the anti-KS MAb AH12; mature chondrocytes (f) and bony tissue (g) with the anti-decorin MAb 6D6; Arrows indicate osteoid. (a) for negative staining and (b) x 105; others x 260.



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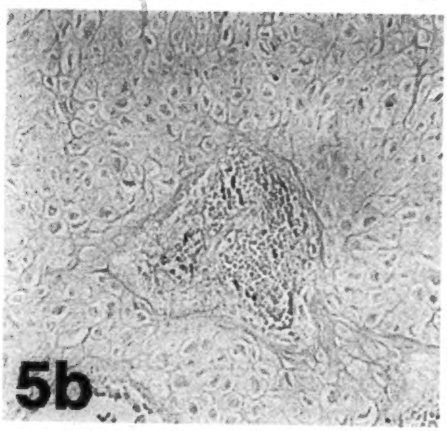

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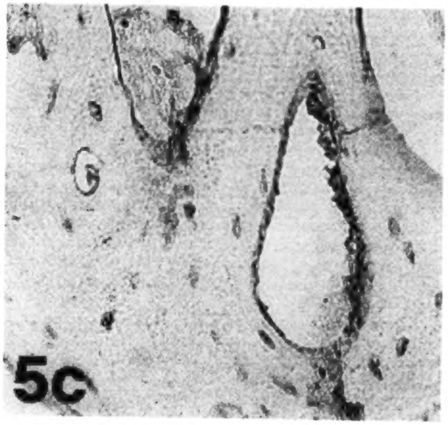

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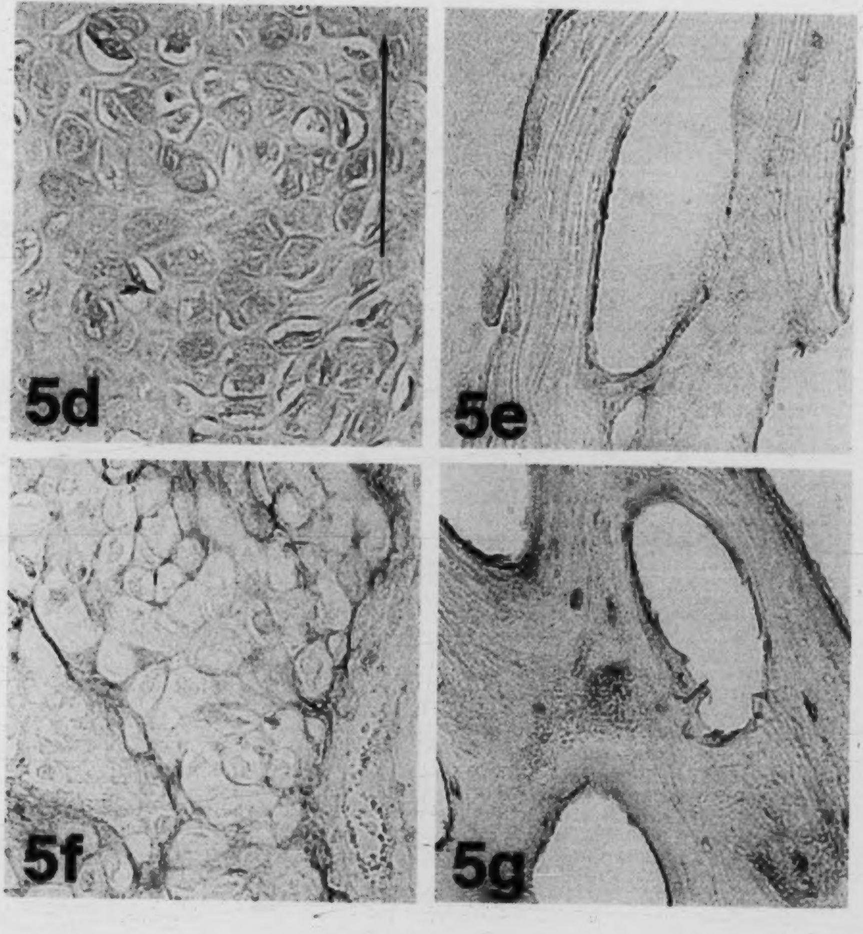
5d
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Chapter 8.

Isolation and Characterization of Proteoglycans in Growing Antlers of Wapiti (*Cervus elaphus*)[1]

8.1. Introduction

The antlers from Cervidae offer unique model of study on bone formation and regeneration among mammals. Following the button-casting of previous sets of hard antlers, regrowth of stag antlers is initiated at the pedicle attached to the frontal bone. The proliferation and differentiation of mesenchymes in distal tip section of antler are developed into chondrocyte which are eventually replaced by osseous tissues in 4 months (Goss 1983). The growth of antler depends on the endochondral ossification in length and intramembranous ossification in diameter as shown in epiphyseal growth plate of long bones (Banks 1974).

The cartilage matrix of the growth plate contained predominantly type-II collagen, together with a large proteoglycan aggregated with hyaluronic acids (Poole and Rosenberg 1987). The matrix in rapid growing antler has been examined by histology. Since the tissue contained sulfated GAGs (Wislocki et al. 1947), they involved in calcification of chondrocytes in developing antler (Mollelo et al. 1963). Frasier et al. (1975) histochemically localized and partially characterized hyaluronic acid and CS in growing antlers. Chemical assays have shown components such as collagen and sulfated glycosaminoglycans (GAG) in the cartilaginous and bony portions of antler (Sunwoo et al., 1995). Large proteoglycans with chondroitin sulfate (CS) and keratan sulfate (KS) were found from the distal tip section of antler (Sunwoo and Sim 1996).

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[1] *A version of this chapter has been submitted for publication. HH Sunwoo and JS Sim 1997. Comp. Biochem. Physiol. (submitted).*

Proteoglycans are macromolecules in extracellular, although both intracellular and cell membrane proteoglycans have been found. The study of proteoglycans dates back to the middle of 19th century. Those early investigations were initiated in the isolation of chondroitin sulfate from cartilage tissues. In the early 20th century, structures of glycosaminoglycans (termed as mucopolysaccharide) were extensively established by the work of Karl Meyer and his colleagues. Since then, study on the function of proteoglycan was initiated after the finding of the formation of the supramolecular complexes with hyaluronan in cartilage tissues (termed as large aggregated cartilage proteoglycans, aggrecan).

The functions of proteoglycans is known to regulate diffusion and flow of macromolecules through connective tissues. In cartilage tissues, proteoglycans also provide resilience because of their polyanionic character of glycosaminoglycans binding other molecules, such as cationic ions, peptides and proteins. Thus, with respect to extracellular matrix assembly, cell adhesion, cell migration, polarization, gene regulation, proliferation and differentiation, proteoglycans with carbohydrate moieties are functionally and metabolically important to the growth of antler.

As matrix organizers, proteoglycans are crucial proteins that are covalently linked with at least one GAG chain (Ruoslahti 1988). The sulfated GAGs in the form of proteoglycans function to hold water in extracellular matrix as well as to interact with many other molecules such as fibronectin, collagen, laminin and growth factors (Hardingham and Fosang 1992). In the development of antler maturation, the molecules might be important for the endochondral ossification (Hunter 1991).

However, little is known about proteoglycans in growing antlers. Understanding the component is, therefore of basic importance related to the study on the biological significance of chondrogenesis and osteogenesis in growing antlers. In the present study, proteoglycans from the zone of maturing chondrocytes have been isolated and partially characterized.

8.2. Materials and Methods

Tissues

Three sets of growing antlers of three four-year-old wapiti (*Cervus elaphus*) were obtained from the University of Alberta Ministik Research Station. The growing velvet antlers were harvested about 60 - 65 days after casting off the buttons from the previous set. Care of animals and procedures used was in accordance with the guidelines of the Canadian Council on Animal Care (1993). Distal portion of the antler beam were cut longitudinally, fixed in 4% buffered formalin (pH 7.3 containing 0.5% cetylpyridium chloride) and decalcified in 22.5% formic acid and 10% sodium citrate. After dehydration, the specimens were embedded in paraffin and sectioned at 5μm. Light microscope showed similar distribution of cell types as previously reported by Banks (1974). Major tissues used in the present study contain most mature chondrocytes with primary spongiosa as a minor tissue undergoing endochondral ossification.

Extraction of Proteoglycans

The antler tissues were skinned, rinsed with cold distilled water to remove blood and finely diced into approximately 1 mm^3 cubes with a scalpel. Proteoglycans were extracted with ice-cold 10 volume of the buffer containing 4 M guanidine HCl, 50 mM Tris, 50 mM sodium acetate, and proteinase inhibitors such as 10 ml ethylenediaminetetraacetate (EDTA), 10 mM benzamidine HCl, 100 mM 6-amino-n-hexanoic acid, 5 mg leupeptin, 5 mg pepstatin, 0.5 mM N-ethylmalemide, and 1 mM phenylmetanesulfonyl fluoride, pH 6.5. Tissues were extracted by slow stirring at 4°C for 48 h and then the extracts were filtered through cheese cloth at 4°C. Contents of uronic acid were measured in both the guanidine HCl-extract and the papain digest of the residue. Extracts were then concentrated by using an Amicon concentrator (Amicon Corp., Danvers, MA 01923, USA) under nitrogen gas at 4°C. The 10 times concentrated supernatant was dialyzed against 20 volumes of 7 M urea, 50 mM Tris, 20 mM NaCl, 10 mM EDTA, pH 6.5, containing proteinase inhibitors as same above at 4°C for 24 h.

Ion-Exchange Chromatography

The dialysates (approximately 360 ml) were applied to a 2.5 x 20 cm DEAE-Sephacel column equilibrated with the 7 M urea buffer at 4°C. Unbound molecules were washed through the column with 2-3 volumes of the same buffer. The column was then eluted with a linear gradient of 0.15 to 1.0 M NaCl in the same buffer. After the gradient elution, the column was washed with 2.1 M NaCl solution in the same buffer. Fractions of approximately 10 ml were collected and aliquot of each fraction was monitored for uronic acid and sulfated GAG contents and for absorbance at 280 nm to estimate protein contents. Fractions containing proteoglycan were pooled and concentrated with an Amicon concentrator under nitrogen at 4°C. Approximately 420 ml of major eluates from DEAE-Sephacel column were combined and concentrated up to 30 ml for further isolation.

Sepharose CL-4B Chromatography

The eluates were applied to a column of gel chromatography on Sepharose CL-4B carried out on a 2.5 x 110 cm column equilibrated with 7 M urea, 50 mM Tris HCl, and 0.5 M sodium acetate, pH 6.5 at 4°C. Fractions of approximately 5 ml were collected and assayed for uronic acid and sulfated GAG contents. Protein in each fraction was also monitored by measuring absorbance at 280 nm. Fractions containing proteoglycan were pooled, and dialyzed against deionized water at 4°C for 24 h. Dialysates were then freeze-dried for further studies.

Cellulose Acetate Electrophoresis

Electrophoresis of GAGs on cellulose acetate strips (Gelman Sciences) was carried out in 0.1 M pyridine/1.2 M acetic acid, pH 3.5 (Habuchi, et al. 1973). Strips were stained in 0.1% (w/v) Alcian Blue 8GX in 0.1% acetic acid containing 0.02% sodium azide for 3 min, washed in 0.1% acetic acid, and scanned at 600 nm on a Gilford-252 spectrophotometer fitted with a linear transporter.

Enzymatic Digestion of GAG Fractions

The residue of guanidine-HCl extracts was digested with twice-crystallized papain (Sigma Chemical Co.) as described (Scott, 1960). The uronic acid of papain digests was determined. Digestions with chondroitinase-ABC and ACI (both obtained from Seikagaku America Inc. Rockville MD, U.S.A.) were carried out following the procedure of Saito et al. (1968). The digests were electrophoresed on cellulose acetate (see above). The digestibility on cellulose acetate electrophoresis was estimated by comparing peak areas obtained after scanning electrophoretograms for samples incubated with and without enzyme. All enzymes used were tested beforehand for activity against standard samples of the appropriate GAGs: human umbilical cord hyaluronic acid (Sigma Chemical Co.), whale cartilage CSA, shark cartilage CSC (both from Miles Laboratories), and hog skin DS (ICN Biomedicals).

Gel electrophoresis and Immunoblotting

Electrophoresis was carried out in 5% polyacrylamide slab gels in Tris-borate-SDS buffer, pH 8.6 (Pringle et al. 1985). Samples were dissolved in 40 mM Tris-borate, 6 M urea, 10% (v/v) 2-mercaptoethanol and boiled for 5 min prior to application. Two gels were generally run at the same time, one for staining with Toluidine blue or Coomassie blue and the other for immunoblotting with monoclonal antibodies (MAbs) to chondroitin sulfate (CS-56; Avnur and Geiger 1984), to keratan sulfate (AH12; Nakano et al. 1993) or to decorin (6D6; Pringle et al. 1985). Core protein samples for electrophoresis were prepared from intact proteoglycans by incubating with chondroitinase-ABC (Pringle et al. 1985). Electrophoretic transfer to nitrocellulose was accomplished in Tris-borate gel electrophoresis buffer without SDS at 40 volts for 2 h. Nitrocellulose sheets were then soaked in 2% bovine serum albumin (BSA) in phosphate buffered saline (PBS), pH 7.2 for 1 h at room temperature. After washing in PBS (three times for 5 min each time) nitrocellulose sheets were incubated separately with MAbs, CS-56 (1:250 in PBS), AH12 (1:250 in PBS), and 6D6 (1:10 in PBS) for 1 h at room temperature. This was followed by washing in PBS, and incubation for 1 h with rabbit anti-mouse IgM for CS-56 and AH12 MAbs and IgG for 6D6 MAb. IgM and IgG conjugated with horseradish peroxidase (1:1000 dilution in PBS with 1% BSA; Sigma) were used as secondary antibodies. Color was developed by incubating in 0.05% diaminobenzidine tetrahydrochloride (Sigma) in PBS containing hydrogen peroxide (0.01% w/v) and cobalt chloride (0.033% w/v) for 5 min. Stained blots were then washed several times in water and then dried.

Interaction of Proteoglycan Fractions with Hyaluronic Acid

To determine the interaction of proteoglycans with hyaluronic acid from each fraction, gel chromatography on Sepharose CL-2B was carried out on a 0.7 x 70 cm column equilibrated with 0.5 M sodium acetate buffer (pH 6.8). Hyaluronic acid (high molecular weight from sigma) was added at a final concentration of 1% (w/w) of the total content of proteoglycans, and then incubated in the elution buffer (pH 6.8) for 12 h at 4°C. Fractions of approximately 0.5 ml were collected and assayed for uronic acid content. The content of proteoglycan in the sample was estimated as being four times the content of uronic acid (Santer et al. 1982).

Analytical Methods

Amino acid analysis was carried out as previously described (Sunwoo et al. 1995). Protein content was measured by the method of (Lowry et al. 1951) with BSA as a standard. Uronic acid was determined by the carbazole reaction (Kosakai and Yosizawa 1979) using D-glucuronolactone as a standard. Sulfated GAG content was estimated by the dimethylmethylene blue (DMB) dye binding method (Farndale et al. 1982). Chondroitin sulfate A from whale cartilage (Miles Laboratories) was used as a standard GAG.

8.3. Results

From light microscopic observation, tissues used in the present study comprised of columns of clustered chondrocytes containing extensive blood vessels. The mature chondrocytes adjacent to perivascular space appeared to be calcified. These tissues were extracted by 4 M guanidine HCl buffer. Determination of uronic acid was made in guanidine HCl extracts and tissue residues. Extractability was approximately 88% of the total that was determined.

The dialysates of the guanidine HCl-soluble fraction against 7 M urea solution obtained from three antlers applied to a column of DEAE-Sephacel and then two peaks were eluted in the range from 0.25 to 0.43 (minor fractions) and from 0.44 to 0.9 M (major fractions) NaCl gradient (Figure 8.1.). The minor fraction had the electrophoretic mobility identical to that of hyaluronic acid on cellulose acetate and was completely digested with *Streptomyces* hyaluronidase, an enzyme which is specific to hyaluronic acid (Ohya and Kaneko 1970) (results not shown). This minor fraction containing hyaluronic acid was not examined further in this study. The major fraction determined by uronic acid content was eluted as a single peak. The proteoglycan fractions were applied to a column of gel chromatography on Sepharose CL-4B. The major proteoglycan-containing three fractions, I, II and III were obtained from the column as the determination of uronic acid and sulfated GAG content (Figure 8.2.).

The general composition in the three proteoglycan Fractions is shown in Table 8.1. The ratio of protein and uronic acid in each fraction was 1:4, 1:1.6, and 1:0.76 in Fractions I, II, and III, respectively. The amino acid analysis showed high amounts of glutamic acid and glycine and lesser, but relatively high levels of aspartic acid, serine and leucine in three fractions. Fraction I containing large proteoglycans had high contents of glutamic acid, serine, and glycine, while Fraction III containing small proteoglycans had relatively high contents of aspartic acid, glutamic acid, glycine and leucine. Fraction II showed intermediate contents of amino acids between Fractions I and III.

Each proteoglycan fraction was separately digested with the specific enzymes chondroitinase ABC and ACI. The susceptibility of each fraction to either enzyme was evaluated by cellulose acetate electrophoresis (Results not shown). The assay gave one single band, of which mobility was faster in Fraction I than in either Fractions II or III. Fractions I and II contained CS highly susceptible to chondroitinase ACI indicating no iduronate-containing polysaccharides, while fraction I showed 92% digestibility with small amounts of chondroitinase ACI resistant materials. The band in Fraction III disappeared after chondroitinase ABC digestion suggesting the presence of dermatan sulfate (DS) proteoglycan.

Molecular weight of proteoglycans with and without chondroitinase-ABC digestion was determined by 5% SDS-polyacrylamide gel electrophoresis (Figure 8.3.). Upon chondroitinase-ABC digestion the Fraction III had a band with apparent molecular weight of 48,000 in the gel stained with Coomassie blue (Figure 8.3.A lane 2). Chondroitinase ABC treated proteoglycans of Fractions I and II were not stained by Coomassie blue in the present study (results not shown). Intact proteoglycans in Fractions I and II did not penetrate into the gel, but showed weak staining with Toluidine blue (Figure 8.3.B lane 4 and 5) compared to the aggrecan purified from bovine nasal cartilage (lane 3). While Fraction III contained small proteoglycans with a molecular weight 160,000 in the gel stained by Toluidine blue (Figure 8.3.B lane 6).

The immunoblotting study showed that both MAbs, CS-56 (Figure 8.4.A) and AH12 (Figure 8.4.B), were reactive to glycosaminoglycans of proteoglycans from all Fractions. Proteoglycans in Fraction I also gave a blot for either MAbs in the same position as the band staining with Coomassie blue. The size of band was broader in Fraction II and broadest in Fraction III than in Fraction I in either MAbs. The MAb 6D6 which was raised against bovine skin decorin (Pringle, et al. 1985) showed a positive reaction only in the band from Fraction III (Figure 8.4.C lane 4), indicating the presence of decorin in this fraction.

Aggregation of proteoglycans with added hyaluronic acid was examined by gel chromatography on Sepharose CL-2B. The large proteoglycan in Fraction I showed an aggregation capability of about 40% (Figure 8.5.d). This was lower than the proportion (54%) of bovine nasal cartilage aggrecan that could interact with hyaluronic acid (Figure 8.5.b). Approximately 5% aggregation in Fraction II was observed (Figure 8.5.f). There was no evidence for the interaction of the small proteoglycans (Fraction III) with hyaluronic acid (Figure 8.5.h).

8.4. Discussion

We suggest that the zone of maturing chondrocytes where endochondral ossification occurs appear to contain two subpopulations of a large proteoglycan which can aggregate with hyaluronic acid and a small non-aggregating proteoglycan which is reactive with anti-decorin MAb, 6D6.

The development of antler is similar with elongated process shown in growth plates of long bones (Goss, 1983). During the longitudinal growth of long bone, cartilage growth plate consists of the proliferative zone and hypertrophic zones containing the unmineralized and the calcified portions, respectively. In general, proteoglycans in these zone had a molecular architecture in a form of aggregation with hyaluronic acids (Buckwalter and Rosenberg, 1986). The chondroblasts synthesize type II collagen and aggrecan (Caplan, 1991). Proteoglycans are important for the mechanical properties and for the regulation of calcification of growth plate (physis) of long bones (Poole, 1986). Compared to long bones, the tissue used in this study was similar with hypertrophic zone of growth plate of long bones, except a difference of extensive vascular spaces in antlers.

Antlers share the similarity with hypertrophic zone of growth plate of long bones, but differ in the extensive vascular spaces in antler. From this study, large proteoglycans in Fraction I obtained from Sepharose CL-4B chromatography contained CS and KS through immunoblotting assay using CS-56 and AH12 MAbs. The proteoglycan was highly susceptible with chondroitinase ACI indicating CS as a major GAG. Molecular size of large proteoglycan in fraction I after the enzyme digestion was larger than that of the core protein (225-250 KDa) of aggrecan (Doege 1987). The reason may reflect a difference in the chemical composition which materials undigested by the enzyme contain KS and oligosaccharides in the large proteoglycan in Fraction I.

Large proteoglycans in fraction I obtained from Sepharose CL-4B chromatography contained CS and KS through immunoblotting assay using CS-56 and AH12 MAbs. The fraction I was highly susceptible (100%) with chondroitinase ACI indicating CS as a major GAG. Molecular size of large proteoglycan in fraction I after the enzyme digestion was larger than that of the core protein (225-250 KDa) of aggrecan (Doege 1987). The reason may reflect a difference in the chemical composition which materials undigested by the enzyme contain KS and oligosaccharides in the large proteoglycan in fraction I. In this study, the commercially purified aggrecan from bovine nasal cartilage showed the proportion of 54% of aggregates with hyaluronic acid, but the large proteoglycan (fraction I) aggregated only 30%. The reason for difference of aggregation is unknown.

Aggrecan, a representative large aggregating proteoglycan in cartilage, is covalently attached with over 100 CS chains, about 20-50 KS and O-linked as well as N-linked oligosaccharides (Heinegård et al. 1985) containing a hyaluronic acid binding region in its protein core (Heinegård and Sommarin 1987). The aggrecan with abundant anionic hydrophilic CS chains (accounting for approximately 90% of dry weight) is thought to provide compressive strength of the load bearing cartilage (Hardingham and Fosang 1992). Due to the CS chains, aggrecans are also suggested to regulate differentiation and proliferation of chondrocytes (Goetinck 1982; Hardingham and Fosang 1992; Maurer et al. 1994), and endochondral ossification (Hunter 1991), which is a process involved in longitudinal growth of antler (Banks 1974). Thus, the large proteoglycan in fraction I with abundant CS found in present tissue, which are apparently subjected to minimum load appear to reflect the importance in both cell growth and endochondral ossification in growing antlers.

However, there is little information about the presence of smaller proteoglycans such as biglycan or decorin in growth plate (physis). Bosse et al. (1993) localized decorin in the mineralization zone of the human growth plate, but did not in the growth plate of chicken tibia (Nakano and Sim 1995). Recently we have immunostained decorin in the osteoid and the zone of maturing chondrocytes with 6D6 MAb (unpublished data H.H. Sunwoo and J.S. Sim). The similarities between decorin and low molecular weight proteoglycan in fraction III obtained from maturing chondrocytes of antler were found in the present study. Composition of the core protein was greater in leucine level in the small proteoglycans (fraction III) than in the large proteoglycan (fraction I). The apparent molecular weight of protein core in proteoglycans digested by chondroitinase ABC was 48,000 in small proteoglycans (fraction III) on 5% gel electrophoresis, while that of the intact proteoglycan gave one broad band showing 160,000 Mr. In addition to that, 6D6 anti-decorin MAb reacted with only fraction III convincingly indicating the presence of decorin. The small proteoglycan in fraction III was highly susceptible to chondroitinase ACI, but had a small amount of material resistant to the enzyme. The resistant material was completely digested by chondroitinase ABC indicating iduronate-containing GAG (DS).

Decorin carries one single dermatan and/or chondroitin sulfate chain (Heinegård and Oldberg 1989). Choi et al. (1989) reported that decorin contains a single chain of DS/CS copolymer in bovine cartilage, whereas in bone it is substituted with CS chain (Franzén and Heinegård 1984; Scott and Haigh 1985). The KS of fraction III recognized by AH12 might be derived from the degradation products of large proteoglycans. The copolymer of CS and DS in decorin of this tissue remains to be further determined. The major function of this molecule is known to bind to interstitial collagens and inhibit fibrillogenesis of collagens (Scott et al. 1981; Poole 1986). Decorin is a low molecular weight proteoglycan (Mr ~100,000), which can bind to transforming growth factor (TGFß) in vitro, suggesting to serve as local tissue reservoirs for this growth factor (Kresse et al. 1994). The role of decorin in vivo is unknown but it has been suggested that decorin may function to inhibit mineral deposition within the collagen fibrils of normally calcified tissues (Scott and Haigh 1985). There is little information available on the role of decorin in antlerogenesis by controlling the activity of TGFß which stimulates collagen (Fine and Goldstein 1987) and proteoglycan (Chen et al. 1987) synthesis. We propose that the presence of decorin in maturing chondrocytes in a growing antler may be important for above suggestions.

Thus, proteoglycans found in the present study may provide a basic information on matrix structure of chondrocytes and endochondral ossification in growing antler. We suggest that large aggregated proteoglycan and decorin in the zone of maturing chondrocytes of growing antler might be important molecules involved in the endochondral ossification and cell growth, although the interaction of proteoglycans with collagen and/or non-collagenous proteins and their roles in a growing antler during the endochondral ossification remain to be further studied.

Table 8.1. Amino acid, protein and uronic acid contents of proteoglycans after Sepharose CL-4B gel chromatography

	Fractions		
Amino acid (μmol/mg)[a]	I	II	III
Aspartic acid	0.67	0.77	1.02
Glutamic acid	1.33	1.25	1.13
Serine	0.90	0.75	0.55
Histidine	0.16	0.21	0.20
Glycine	1.38	1.28	1.08
Threonine	0.55	0.51	0.43
Arginine	0.32	0.29	0.31
Alanine	0.62	0.61	0.52
Tyrosine	0.08	0.07	0.09
Valine	0.69	0.65	0.53
Phenylalanine	0.31	0.30	0.28
Isoleucine	0.32	0.35	0.42
Leucine	0.72	0.85	1.00
Lysine	0.22	0.30	0.46
Protein (μg/mg)[a]	88.3	208.67	221.5
Uronic acid (μg/mg)[a]	353.3	332.5	169
Ratio of Protein:Uronic acid	1:4	1:1.6	1:0.76

[a] dry weight basis.

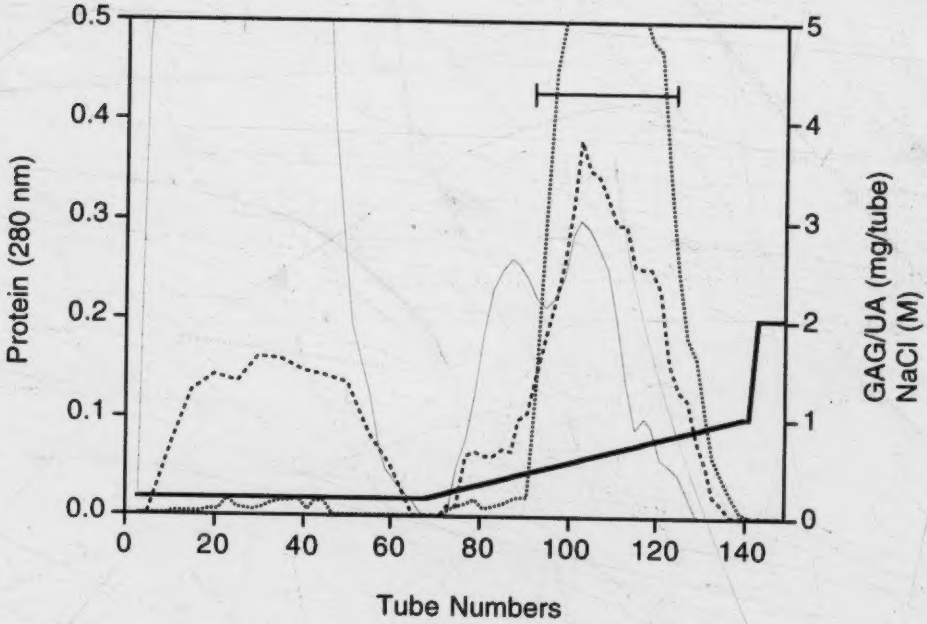


Figure 8.1. Chromatography on DEAE-Sephacel of 4 M guanidine-HCl extracts of a growing antler. Fractions (10 ml) collected were determined for the absorbance at 280 nm (—), the uronic acid content (-----), the GAG content (········) and the gradient (0.15 to 1 M) of NaCl (——). Fractions indicated by the bar were combined for further isolation. Samples were eluted with a total volume of 720 ml at a flow rate of 30 ml/h.

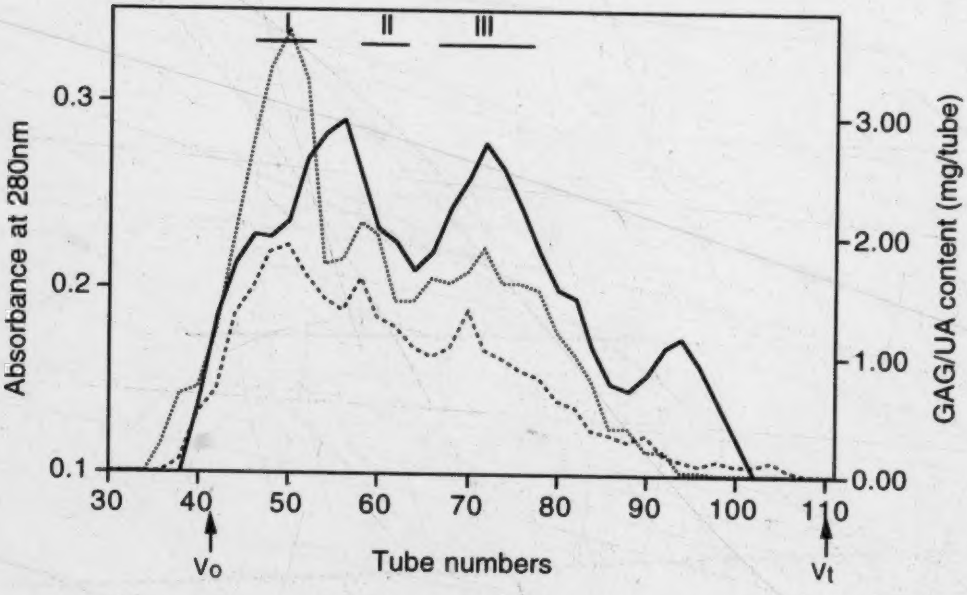


Figure 8.2. Gel chromatography on Sepharose CL-4B of proteoglycans obtained from DEAE-Sephacel column. Fractions (5 ml) were determined for the absorbance at 280 nm (—), the uronic acid content (-----), and the GAG content (·········). V_o and V_t show positions of excluded and total column volumes, respectively. Bars denote fractions pooled for further study. Samples were eluted at a flow rate of 15 ml/h.

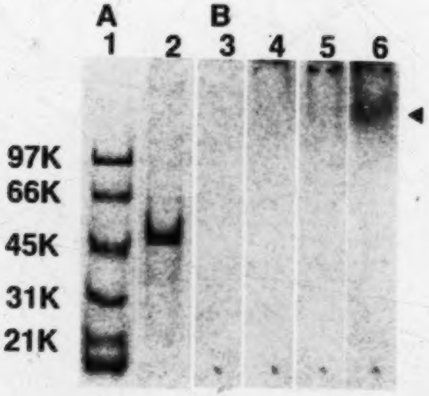


Figure 8.3. Gel electrophoresis of three proteoglycan fractions from a growing antler. A) Coomassie blue-stained 5% SDS-polyacrylamide gels of chondroitinase ABC digestion: Reference of low molecular weight proteins is shown in lane 1. Chondroitinase-ABC digested proteoglycan fraction III is shown in lane 2. B) Toluidine Blue-stained 5% SDS-polyacrylamide gels of fractions (I, II, and III): Aggrecan from bovine nasal cartilage (ICN Biomedicals) is negatively stained in lane 3. Fractions I and II are shown in lane 4 and 5, respectively. Fraction III shows the dermatan sulfate proteoglycan in lane 6, which appears as a broad and heavily stained band of 160,000 molecular size marked by arrow head.

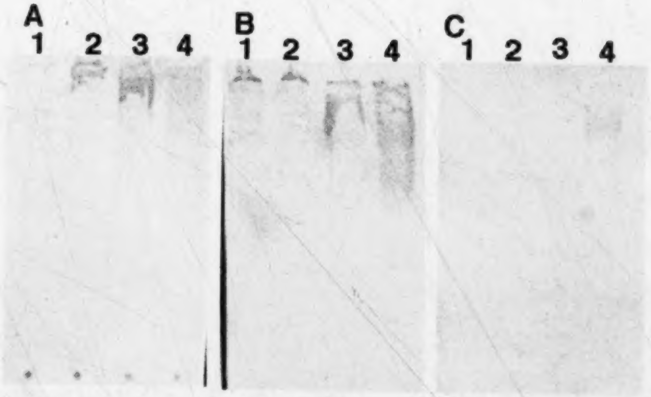
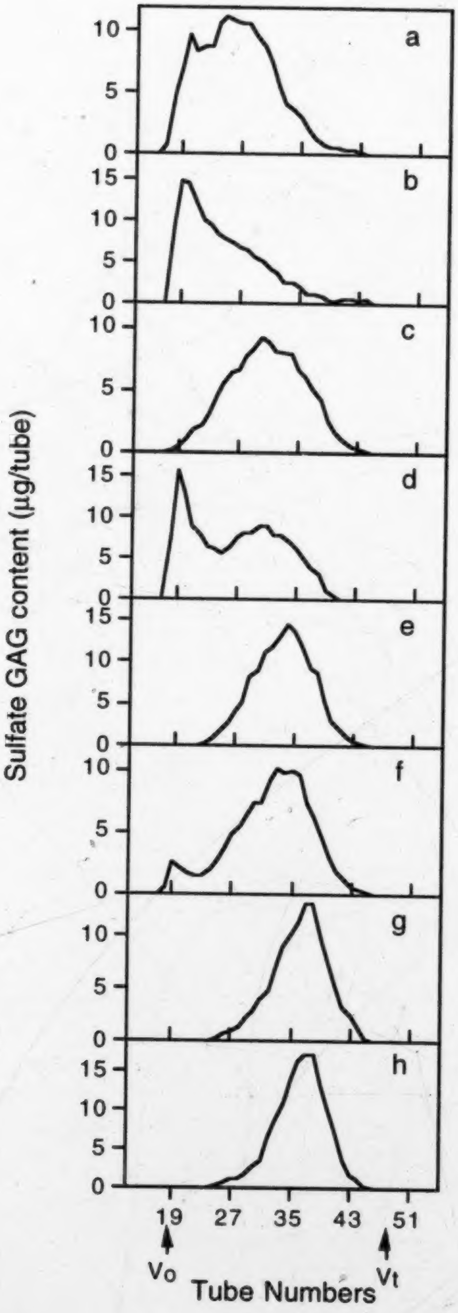


Figure 8.4. Immunoblotting of proteoglycan fractions.
(A) Stained with MAb to chondroitin sulfate (CS-56); (B) Stained with MAb to keratan sulfate (AH12); (C) Stained with MAb to decorin (6D6). Lanes 1 (aggrecan from bovine nasal cartilage), 2 (Fraction I), 3 (Fraction II) and 4 (Fraction III).

Figure 8.5. Gel chromatography on Sepharose CL-2B under associative conditions of proteoglycans. Approximately 300 µg of uronic acid in proteoglycan fractions was incubated with 12 µg of hyaluronic acid in 0.01 M Tris-HCl, pH 6.8, for 18 h at 4°C. The fractions (0.5 ml) were monitored for uronic acid content. Controls (a, c, e, and g) were incubated without hyaluronic acid. V_o and V_t show positions of excluded and total column volumes, respectively. Samples were eluted at a flow rate of 4 ml/h.


a
b
c
d
e
f
g
h
Sulfate GAG content (μg/tube)
0
5
10
15
19
27
35
43
51
Vo
Vt
Tube Numbers

8.5. References

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Chapter 9.
General Discussion and Conclusions

There have been extensive efforts by many scientists for the past decades to determine the curious effects of velvet antler on physiological growth and pharmacological efficacy in the West and Orient, respectively. However, we have to admit that no complete understanding has been made, even though many answers on the mystic phenomena have been explained in various areas. To answer these apparently difficult questions and many more, the research to explain both unique biological and pharmacological activity must be done. This thesis began with indefinite guess, showed the possible development of a value-added product partially investigated and provided information on biologically active components in velvet antler. Although there are our findings on the research of antler, the study of biological or pharmacological activity in velvet antler must be investigated further.

First of all, research of velvet antler is becoming increasingly important for the deer industry. World wide, major antler producers including North America, Russia, China and New Zealand account for most of supply in the international market to supplement local production in Eastern Pacific Rim Countries. Among importers, Korea as major player accounting for 85% of the total amount of antler in the international markets is accelerating the consumption of velvet antlers with economic growth for the past 10 years. According to the report of Korean Trade Institute, Korea imported 21 tonnes of antlers from the international market in 1985, since then it increased by about 700% in 1994 (163 tonnes). It is expected the importing amount of velvet antlers reach about 200 tonnes in 1997. However, the demand may be falling due to the current economic downturn. In order to expand the market from the eastern culture to the western culture, the development of antler products as a modern nutraceutical appears significant. Despite the current economic opportunity, prospects for developing a western nutraceutical market has been dampened by incomplete understanding of the chemical and pharmacological properties of velvet antler. Such information is important to expand the antler industry as understanding antler function as nutritional supplement or pharmaceutical agents.

This thesis provided basic information on the morphology and chemical composition of growing antlers which are commercially available. Wapiti antlers at 60-65 days of growth were histologically examined by light microscope (Chapter 2). The distal tip section contained reserve mesenchymes adjacent to the dermis, prechondroblasts, chondroblasts and immature chondrocytes as shown in other reports (Banks 1974; Banks and Newbrey 1983). Zone of maturing and calcifying chondrocytes referred as upper section in this thesis was invaded by osteoblasts indicating the occurrence of endochondral ossification with consistent observation of Banks (1974) who reported that antler growth undergoes both endochondral ossification and intramembranous ossification. The cartilaginous tissues were gradually replaced by osseous tissues downward. The bony tissues referred as the middle and base sections in this thesis contained spongy bone and cortical bone structure in the difference of the degree of mineralization and the thickness of cortical bony in adjacent to outer velvet layer.

In the Orient, different sections of antler have been used for particular purpose; cartilaginous tissues equivalent to tip and upper sections have traditionally been used for children and young people as a preventative medicine, the mineralizing middle sections have been used in the treatment of arthritis and osteomyelitis, while the mineralized base section in old people lacking in calcium (Fennessy 1991). Market prices of velvet antlers are different from sections. The price of cartilaginous portion is approximately 10 times higher than that of bony portion in the velvet antler. However, the chemical composition of each section has not been elucidated. Also, there is little information available concerning chemical composition of antlers from wapiti (*Cervus elaphus*). In Chapter 3, chemical compositions of antler in different section were determined by the contents of dry matter, collagen, calcium, phosphorus and magnesium which were lowest in the tip section, and increased downward. In contrast, the contents of protein, lipid, uronic acid, sulfated glycosaminoglycans (GAG) and sialic acid were higher in the tip and upper sections than in the middle and the base sections. Collagen has been suggested to be the major protein in the antler (Goss 1983). Our data indicated that the amount of collagen in dry tissue increases downward with concomitant increase in mineral contents. Interestingly, the content of GAG is apporximately 10 times higher in cartilaginous portion (tip and upper sections) than in bony portion (middle and base sections). The molecule may be a good indicator to distinguish different tissues in velvet antler.

The chemical study played a role in the provision of useful information about the biologically active component. It has been proposed that anionic molecules of chondroitin sulfates play physiologically important roles as ion-exchangers in endochondral bone formation (Hunter 1991). Chondroitin sulfate may be a potentially important carbohydrate in the antler as a food (Sunwoo et al. 1995). Chondroitin sulfates are known to reduce pain in osteoarthritis patients (Paroli et al. 1991). Thus, further chemical study focused on the interesting molecule in Chapter 6 to 8.

In addition to that, antler extract as a possible pharmaceutical agent was investigated in Chapter 4. The tip section known as the growth center of antlers contains various growth promoters which are epidermal growth factor (Ko et al. 1986) and insulin-like growth factor (Suttie et al. 1991). We conducted *in vitro* experiment whether water-soluble extract from antler tip sections may contain growth promoter(s) to stimulate the growth of bovine skin fibroblasts. It was suggested that GAG in a form of proteoglycan in water-soluble extract obtained from Sephacryl S-300 column may be a growth promoter. There was not complete but big step for the development of pharmaceutical agents in the antler extract.

Pharmacological efficacy of velvet antlers based on the Oriental preparation indicated that velvet antlers stimulate body performance as a tonic, recover injured tissues, affect immune response, slow the aging process, have hypotensive-vascular effect, and enhance gonadotrophic and thyroid functions. The pharmacological study in Chapter 5 was conducted on changes of cholesterol concentrations and immune responses in growing rats fed antler powder. In the present study, antler powders appear to have effects on the prevention of the risk of coronary heart disease and immunosuppression such as anti-inflammation. These results may stimulate antler industry for the development of antler products as nutraceuticals as well as pharmaceutical agents. We proposed that additional study on the biologically active component related to above results remains to be further investigated.

However, a sulfated glycosaminoglycan investigated partially in Chapter 3 and 4 shows its possible use as pharmaceutical agents and the biological important molecule to understand the antler growth. With the exception of the GAG, hyaluronic acid, which exists as a free polymer, all GAGs including chondroitin sulfate, dermatan sulfate, keratan sulfate, heparan sulfate and heparin are covalently attached to core proteins to form proteoglycans, and have important physiological functions such as water retention and electrolyte control (Hardingham and Fosang 1992). Cartilage proteoglycans regulate differentiation and proliferation of chondrocytes (Goetinck 1982). Mollelo et al. (1963) in a study of developing antler of mule deer (*Odocoileus hemoinus*) suggested GAGs as important molecules involved in calcification of chondrocytes. Thus, GAG and proteoglycans appear to have important roles for the development of antler. Pharmacists are interested in those molecules of which positive effects as pharmacological agents to treat diseases such as arthritis, atherosclerosis, and cancer have been reported elsewhere (Prudden 1985; Matsushima et al. 1987; Dean et al. 1991). Investigations of pharmacological effects of antler GAGs may be important in this regard. Furthermore, chondroitin sulfate was also chemically analyzed in the fossilized deer antler (Scott and Hughes 1981). If GAGs in antlers can be proved as the possible active component, the hard antler might be useful alternative of growing antlers which are highly innervated and vascularized (Woodbury and Haigh 1996) on issues of animal welfare as product quality.

Due to the importance of GAGs in both biological and pharmacological activities, we characterized the GAG and proteoglycans in growing antlers. Glycosaminoglycans were isolated from the tip to the base section of the main beam of growing antlers of wapiti (*Cervus elaphus*). Chondroitin sulfate was found as the major glycosaminoglycan in each section of antler. The yield of chondroitin sulfate liberated from the tissue was approximately six fold greater in the cartilaginous (tip and upper) sections than in the bony (middle and base) sections. The ratio of 4-sulfated unsaturated disaccharide to 6-sulfated unsaturated disaccharide was higher in bony tissues than in cartilaginous tissues The size of chondroitin sulfate was smaller in cartilaginous tissues than in osseous tissues of growing antler. In addition to chondroitin sulfate, the antler contained small amounts of hyaluronic acid, dermatan sulfate and keratan sulfate. In conclusion, the CS is a major GAG localized throughout the main beam of wapiti antlers, but its structure was different quantitatively as well as qualitatively between the cartilaginous (tip and upper) and the osseous (middle and base) tissues. The antler contained, in addition to CS, small amounts of hyaluronic acid, DS, and KS. These findings in Chapter 7 might be interesting to bone biologists and biomedical researchers.

Glycosaminoglycans abundant in cartilaginous portion of velvet antlers always exist in a form of proteoglycan. Proteoglycans are proteins that are covalently linked with at least one GAG chain (Ruoslahti 1988). Aggrecan, a representative large aggregating proteoglycan in cartilage, is covalently attached with over 100 CS chains, about 20-50 KS and O-linked as well as N-linked oligosaccharides (Heinegård et al. 1985) The CS proteoglycans are also suggested to regulate differentiation and proliferation of chondrocytes (Goetinck 1982; Maurer et al 1994), and endochondral ossification (Hunter 1991), which is a process involved in longitudinal growth of antler (Banks 1974). Proteoglycans were characterized in the zone of maturing chondrocytes where endochondral ossification occurs in Chapter 8. The development of antler is similar to that of other long bones (Goss 1983). Proteoglycans are known to be important for the mechanical properties and for the regulation of calcification of growth plate (physis) of long bones (Poole 1986). Thus, proteoglycans (aggrecan and decorin) found in the present study may provide a basic information on matrix structure of chondrocytes and endochondral ossification in growing antler.

In conclusion, this basic information on both pharmacological and chemical characterization of velvet antler is a cornerstone for the use of antler products not only acceptable by the Eastern culture but readily acceptable in the West also. The set of analytical and experimental data of velvet antler generated from this thesis is in dire need for the industry (rancher, processors, traders as well as consumers around the world). Surplus antler stock is already emerging in the traditional world market. This rapidly growing industry can not be merely sustained by a volatile export market alone, but has to be securely founded by a well established domestic market strategy with a diversified antler nutracutical product line. The opportunity for development of a modern nutraceutical market appears vast with strong demand in North America and Europe as well as Asia. Thus, the future antler industry largely depends upon concerted R&D efforts among researchers (Government, Industry and University) by exploring the thousands years old medicinal claims of velvet antlers using modern scientific research tools, and by further processing antler products and developing nutraceutical products.

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Appendices

Appendix 1. Structure of Glycosaminoglycans.

Glycosaminoglycans (GAGs) are an integral part of the proteoglycans with exception of the hyaluronic acid. They were usually extracted from tissues by proteolytic digestion which separate GAGs attached to the core protein from proteoglycans. Much of the structure of GAGs has been elucidated by the work of Meyer et al. (1956). Structures of GAGs shown in this appendix 2 are basically disaccharide units in a linear arrangement.

Disaccharide unit of chondroitin 6-sulfate (a) is derived from the polymer D-glucuronic acid ß1-3 to D-N-acetyl galactosamine; linkage between disaccharide units is ß 1-4. Galactosamine can be selectively sulfated at either the 4 or 6 position. There are two chondroitin sulfates which are chondroitin 6-sulfate (old term; chondroitin sulfate A; GlcUA-GalNAc-6S) and chondroitin 4-sulfate (b) (old term; chondroitin sulfate C; GlcUA-GalNAc-4S). Dermatan sulfate (c) is derived from the polymer D-glucuronic acid ß (1-3) D-N-acetyl galactosamine ß (1-4) (old name; chondroitin sulfate B; IdoUA-GalNAc-4S). Principal difference between dermatan sulfate and chondroitin sulfate is that b-D-glucuronic acid residue in CS is converted to a-L-iduronic acid residue in DS by C_5 epimerization. Repeating disaccharide unit in heparan sulfate (d) is hexuronic acid (either a-L-iduronic acid linked a1-4 to glucosamine (shown) or b-D-glucuronic acid linked b1-4 (not shown). Basic structure of heparin is similar to heparan sulfate but with higher degree of sulfation. In heparan sulfate, amino sugar in 2 position can either be sulfated or N-acetylated. Keratan sulfate (e) is disaccharide unit of b-D-galactose linked b1-4 to N-acetyl-b-D-glucosamine. Linkage between disaccharides is b1-3. This repetitive portion is extremely variable in size. Keratan sulfate in cartilage contains 5 to 10 disaccharide units, while in intervertebral disc 20 to 30, and in corneal 30 to 50 disaccharide units. Hyaluronic acid (f) is only GAG that contains no sulfate groups. It consists of D-glucuronic acid linked b1-3 to N-acetyl-b-D-glucosamine. Disaccharide linkage is b1-4. Hyaluronic acid differs from the other GAGs in that it does not contain any sulfate ester groups.

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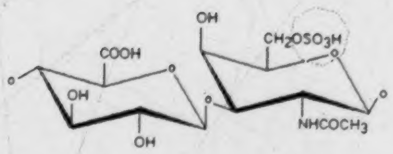
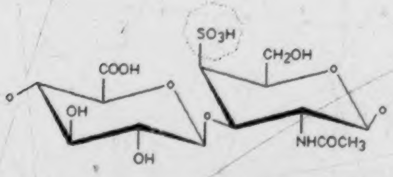
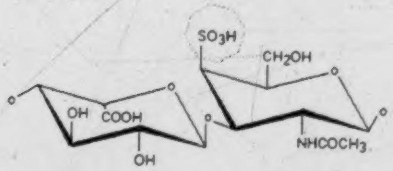

OH
COOH
CH2OSO3H
OH
OH
NHCOCH3

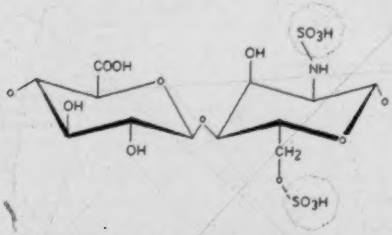
a) Chondroitin 6-sulfate **GlcUA(b1-3)GalNAc(b1-4)**


SO3H
COOH
CH2OH
OH
OH
NHCOCH3

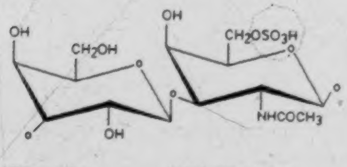
b) Chondroitin 4-sulfate **GlcUA(b1-3)GalNAc(b1-4)**


SO3H
CH2OH
OH
COOH
OH
NHCOCH3

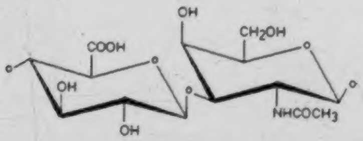
c) Dermatan sulfate **IdoUA(a1-3)GalNAc(b1-4)**


SO3H
NH
OH
COOH
OH
OH
CH2
SO3H

d) Heparan sulfate/Heparin **GlcUA(b1-4)GlcNAc(a1-4)**
or IdoUA(a1-4)GlcNAc(a1-4)


OH
CH2OH
OH
CH2OSO3H
OH
NHCOCH3

e) Keratan sulfate **Gal(b1-4)GluNAc(b1-3)**


OH
COOH
CH2OH
OH
OH
NHCOCH3

f) Hyaluronic acid **GlcUA(b1-3)GlcNAc(b1-4)**

Appendix 2.

Isolation and Analysis of Glycosaminoglycans

Isolation from Cartilaginous Tissue of Growing Antler
(Papain Digestion)

↓

Separation by Charge Density
(Anion Exchange Chromatography)

↓

Dialysis

↓

Ethanol Fractionation

↓

Analysis

Colorimetric Carbazole-Sulfuric Acid Assay
Dye-Binding Assay-Alcian Blue, 1,9-dimethylmethylene Blue, Safranin O
Paper or Thin Layer Chromatography
Electrophoresis
(Cellulose Acetate Electrophoresis/ Polyacrylamide Gel Electrophoresis)
Immunoblotting
Enzymic Fragmentation
High Pressure Liquid Chromatography

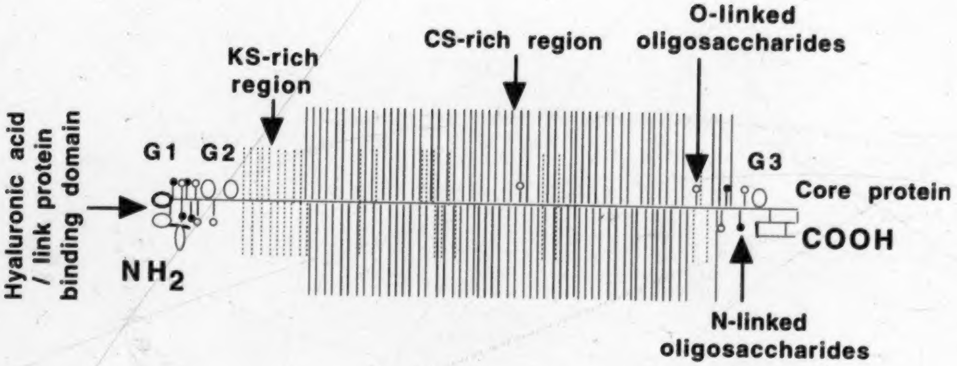
Appendix 3. Structure of Proteoglycans from Growing Antler.

Proteoglycans are complex molecules containing from one or two to several hundred glycosaminoglycan chains covalently attached to a protein core and N- and O-linked oligosaccharides which are found in glycoproteins containing N-acetylglucosamine, mannose, galactose, and sialic acid. The polyanionic GAGs extrude from the central protein core (Poole 1986).

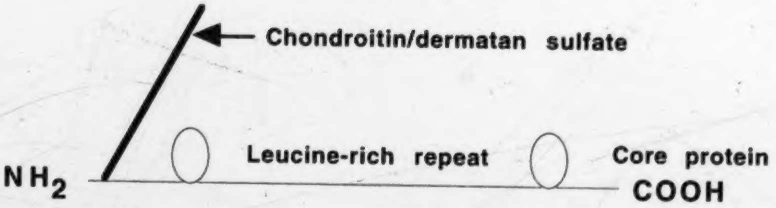
Large chondroitin sulfate aggrecan and small fibroblast decorin are representative of extracellular matrix proteoglycans. The core proteins of these proteoglycans have been cloned (Doege et al. 1991). The former shows a unique property of the hyaluronic acid binding region located at the N-terminus of the core protein. The interaction between hyaluronic acid and aggrecans involves a link protein which exists in the core protein. Extremely large supramolecules aggregated with hyaluronic acid and aggrecan are also organized in collagen fibrils to form matrix in cartilage tissues. More than 100 aggrecan can be arranged on a strand of hyaluronic acid with molecular weight of 1×10^6 which generally forms a complex of molecular weight as large as 2×10^8. Thus, this complex is referred to as aggrecan.

Aggrecan accounts for about 90% of carbohydrate, chondroitin sulfate, keratan sulfate and N- and O-linked oligosaccharides. The core protein contains three globular protein domain: G1 and G2 at the NH_2 terminus, and G3 at the COOH terminus. Aggrecan G1 domain is known to be the member of the immunoglobulin superfamily involved in cell recognition, cell adhesion, or immune function . The G1 domain is responsible for binding hyaluronic acid. However, a role of G2 domain remains unknown. The COOH terminal G3 domain includes three distinct peptide motifs, a complement regulatory protein-like domain, a lectin-like domain and an epidermal growth factor-like domain from COOH to NH_2 terminus, sequentially. Hardingham and Fosang (1992) supposed that the lectin domain with a low-affinity for fucose and galactose ligands may interact with collagen type II and the epidermal growth factor-like domain may have a role in modulating the proliferative activity of chondrocytes and fibroblasts.

Three low molecular weight of proteoglycans-decorin, biglycan and fibromodulin are members of a leucine rich family of proteins. Those core protein size is approximately 40 kDa. Decorin has a chondroitin/dermatan sulfate copolymeric chain. Decorin appears to have an important role in organizing the extracellular matrix, as each bind to collagens types I (Hardingham and Fosang 1992). Decorin can modulate a transforming growth factor ß which stimulates protein core of decorin by the binding of the protein core and the growth factor and neutralize the activity of the growth factor, indicating a negative feedback control (Ruoslahti and Yamaguchi 1991).


O-linked
oligosaccharides
CS-rich region
KS-rich
region
Hyaluronic acid
/ link protein
binding domain
G1
G2
G3
Core protein
COOH
NH2
N-linked
oligosaccharides

Aggrecan


Chondroitin/dermatan sulfate
Leucine-rich repeat
Core protein
NH2
COOH

Decorin

References

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Appendix 4.

Isolation and Analysis of Proteoglycans

Extraction from Cartilaginous Tissue of Growing Antler
(4M Guanidine HCl Buffer with Protease Inhibitors)

↓

Separation by Density
(Sedimentation Equilibrium CsCl Centrifugation)

↓

Dialysis or Rapid Desalt/Buffer Exchange
(Chromatographic Desalting with Sepahdex G-50)

↓

Separation by Charge Density
(Anion Exchange Chromatography)

↓

Separation by Size
(Size Exclusion/Gel Permeation Chromatography)

↓

Analysis

Ultraviolet Absorbance at 210nm or 280nm
Colorimetric Carbazole-Sulfuric Acid Assay
Dye-Binding Assay-Alcian Blue, 1,9-dimethylmethylene Blue, Safranin O
Amino Acid Composition
Electrophoresis
(Cellulose Acetate Electrophoresis/ Polyacrylamide Gel Electrophoresis)
Immunoblotting
Enzymic Fragmentation